INDUCTION OF c-fos AND ACTIVATION OF PARALLEL MAPK CASCADES BY CADMIUM

by

Wei Ding

A thesis submitted in conformity with the requirements for the Degree of Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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ABSTRACT

Cadmium is an toxic metal that can initiate either mitogenesis or apoptosis in different cell lines, probably as a consequence of inducing different patterns of proto-oncogene expression. Previous work in our lab has found that Cd²⁺ can stimulate an increase in c-fos mRNA level in quiescent mesangial cells. Induction of c-fos via one of its regulatory elements SRE can be mediated by at least two major signaling systems, the CaMK pathway and the MAPK cascades. It has been confirmed by the previous study in our lab that the CaMK pathway is not involved in c-fos induction by Cd²⁺. Therefore, we further investigated the involvement of the MAPK cascades. We found that Cd²⁺ caused activation of both ERK and SAPK, and the time courses of ERK activity, SAPK activity, and c-fos mRNA level showed a similar biphasic pattern in response to 10 μM Cd²⁺ with an initial increase at 15 - 30 min and a larger prolonged increase at 8 h. Each of these three signals also showed a similar concentration dependence, different concentrations of Cd²⁺ tested between 0.1 to 10 μM causing the similar initial increases but values above 3 μM required for the prolonged activation. Inhibition of the ERK pathway with the specific MEK1/2 inhibitor PD98059 completely blocked ERK activation by Cd²⁺ but only partially inhibited c-fos induction by Cd²⁺, indicating that the ERK pathway is involved in c-fos induction by Cd²⁺ and additional mechanisms may also be involved in this process. Mesangial cells transfected with dominant negative mutant M KK7-K76E showed greater inhibitory effect on SAPK activation by Cd²⁺ than those transfected with dominant negative mutant SEK1-AL. This observation indicates that SAPK activation by Cd²⁺ is mainly mediated by MKK7 in mesangial cells. Blocking both the ERK and the SAPK pathways with PD98059 and M KK7-K76E respectively showed a synergistic
inhibitory effect on Cd\textsuperscript{2+}-dependent c-fos induction, confirming the SAPK pathway to be another mechanism involved in c-fos induction by Cd\textsuperscript{2+}. The cell viability study showed that mesangial cells lost their viability after exposure to 10 μM Cd\textsuperscript{2+} for more than 8 h. The prolonged activation of ERK and SAPK and the sustained accumulation of c-fos mRNA by 10 μM Cd\textsuperscript{2+} may be associated with this process. Induction of c-fos and activation of ERK and SAPK showed high specificity for Cd\textsuperscript{2+}, while other divalent metals tested under the same conditions (Co\textsuperscript{2+}, Cu\textsuperscript{2+}, Hg\textsuperscript{2+}, Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, Ni\textsuperscript{2+}, and Zn\textsuperscript{2+}) were without any significant effects. Therefore, we conclude that Cd\textsuperscript{2+} is a specific inducer of c-fos in mesangial cells, through activation of both the ERK and SAPK pathways.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>activating protein-1</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CaMK</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>CRE binding protein</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>HSE</td>
<td>heat shock element</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
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<td>IL-2</td>
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</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal protein kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK/MKK</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
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<td>MEKK</td>
<td>MEK kinase</td>
</tr>
<tr>
<td>MKP</td>
<td>MAPK phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MIT</td>
<td>tetrazolium salt</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PD98059</td>
<td>2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>RMC</td>
<td>rat mesangial cell</td>
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<td>RT-PCR</td>
<td>reverse transcription-PCR</td>
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<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>SEK</td>
<td>SAPK kinase</td>
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<tr>
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<td>Src-homology domain 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology domain 3</td>
</tr>
<tr>
<td>SIE</td>
<td>sis-inducible element</td>
</tr>
<tr>
<td>SIF</td>
<td>sis-inducible factor</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>Stat</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TCF</td>
<td>ternary complex factor</td>
</tr>
<tr>
<td>TEY</td>
<td>Thr-Glu-Tyr</td>
</tr>
<tr>
<td>TGY</td>
<td>Thr-Gly-Tyr</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl phorbol-13-acetate</td>
</tr>
<tr>
<td>TPY</td>
<td>Thr-Pro-Tyr</td>
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1. INTRODUCTION

Cadmium is an important environmental pollutant showing genotoxicity and carcinogenicity in some tissues, and non-malignant chronic toxicity to others [1, 2]. Cadmium compounds induce lung tumors in humans and experimental animals after inhalation [3], and tumors of the prostate and testis in rats after subcutaneous injection [4]. However, chronic accumulation of cadmium in the kidney causes progressive nephrotoxicity with no increased incidence of renal cancer [5, 6]. Since cadmium has been reported to interact with cell surface receptors [7], increase intracellular free Ca\(^{2+}\) concentration [8], affect intracellular Zn\(^{2+}\) homeostasis [9], stimulate protein phosphorylation [10] and modify transcription factors [11], these different effects of cadmium may result from interference with intracellular signaling systems.

Cadmium has been known to stimulate the expression of the proto-oncogenes c-fos, c-jun, and c-myc, also termed immediate early response genes in different cell lines [12]. Andrews et al. [13] were the first to show that cadmium induced c-fos in HeLa cells. Other authors reported that cadmium induced expression of c-jun and c-myc in L6 myoblasts [14]; c-jun, c-fos, and c-myc in NRK cells [15]; c-fos and egr-1 in Swiss 3T3 cells [16]; and c-jun, c-fos, c-myc, and egr-1 in rat LLC-PK1 cells [17]. Previous work in our lab has also found that Cd\(^{2+}\) is a transcriptional activator of c-fos in mesangial cells [18]. Since c-fos is generally regarded as a mitogenic signal [19], cadmium has the potential to act as a mitogenic stimulus in some cell lines.

Cadmium can also induce apoptosis in isolated T lymphocytes [20] and cultured LLC-PK1 cells [17] and cause apoptotic cell damage in canine proximal tubules [21] and rat testicular tissue [22]. Numerous authors have also described an association of c-fos expression with apoptosis [23]. Overexpression of c-fos in transfected fibroblasts increased the apoptotic response to serum deprivation by 10-fold [24]. Therefore, cadmium appears to be an interesting agent to explore the factors determining the balance between mitogenesis and apoptosis. Investigating the mechanisms of c-fos induction by cadmium may further elucidate the molecular mechanisms regulating cell fate.
with respect to proliferation or apoptosis.

1.1. Cadmium

1.1.1. Physical and chemical properties of cadmium

Cadmium is a metal that belongs, together with zinc and mercury, to group IIb in the Periodic Table with a completed 4d shell of electrons. Its atomic number is 48 and relative atomic weight is 112.40. It was first discovered by two German investigators, Strohmeier and Hermann, independently and almost simultaneously in 1817 [25], as a constituent of the zinc ore smithsonite (ZnCO₃). Pure cadmium is a bluish-white metal but does not occur as such in nature. The physical and chemical properties of cadmium has been reviewed by Aylett [26]. In chemical reaction, up to two electrons may be removed to form cations. The most common oxidation state for cadmium is Cd(II). Although it has proved possible to isolate a few compounds which formally contain Cd(I), the Cd⁺ ion is extremely short-lived in solution, formed as an intermediate in the reduction of Cd²⁺ to cadmium metal. The ionic radius of Cd²⁺ is 0.97 Å, similar to that of Ca²⁺ (0.99 Å). However, the former ion is more polarizable (because of the larger number of electrons) and more polarizing (because of the relatively poor nuclear screening afforded by the 4d shell). This implies that cadmium compounds, especially those with polarizable ligands, will have an appreciable covalent character. It has been pointed out in some reviews [26, 27] that Cd(II) interacts strongly with a wide range of biological molecules, such as proteins, phospholipids, purines, porphyrins, nucleic acids and enzymes. It is believed that cadmium is attached to these molecules via thio groups in cysteinyl residues, imidazole groups in histidinyl residues, and phosphate groups [25].

1.1.2. Cadmium in the environment

Cadmium is a relatively rare element. Its crustal abundance is estimated between 0.1 μg/g and 0.2 μg/g [28], which lies in 67th position in an order of relative abundance. However, cadmium is widely distributed in the earth's crust and is a natural component of rocks, sediments, soils, waters, air, and plant and animal tissues [29]. Volcanic activity is a major natural source of
cadmium release into the atmosphere. The annual global flux from this source is estimated to be 100 - 500 tonnes [30]. Atmospheric concentrations of cadmium in remote areas are typically in the range of 0.01 - 0.04 ng/m³ [2]. Deep sea volcanism is a source of environmental cadmium release into the marine waters. In water from the open sea, the cadmium concentration varies between 0.02 and 0.1 μg/L [31]. Cadmium concentrations in coastal waters and fresh waters are typically less than 0.1 μg/L, but concentrations exceeding 1 μg/L have been reported in waters obtained from areas where there are cadmium-bearing and zinc-bearing mineral formations [31]. In soils and rocks, cadmium concentrations vary widely, usually in concentrations of less than 1μg/g, but soils derived from some marine black shales and from rocks containing lead-zinc minerals frequently contain much higher amounts [29]. In foodstuffs, cadmium concentrations are commonly below 0.1 μg/g fresh weight. Milk, eggs, beef and fish normally contain less than 0.01 μg/g [31]. Somewhat higher concentrations, ranging from 0.01 - 0.1 μg/g, are usually found in fruits, vegetables, and grains. Oysters, mussels, and certain species of wild white mushrooms often have cadmium concentrations exceeding 1 μg/g [32]. Particularly high concentrations of cadmium, 2 - 30 μg/g fresh weight, are found in the brown meat of marine shellfish [32].

Anthropogenic emissions account for the major part of cadmium release into the environment [2, 29, 31]. With the steady increase of production and consumption of cadmium in the past 50 years, emissions of cadmium into the environment continue to rise. The worldwide anthropogenic emissions into the atmosphere were estimated to be about 7000 tonnes in 1983 [33]. The principal applications of cadmium in industry fall into five categories: protective plating on steel, stabilizers for polyvinyl chloride (PVC), pigments in plastics and glasses, electrode material in nickel-cadmium batteries, and as a component of various alloys [29, 31]. The manufacture and use of these products give rise to the release of cadmium into the environment in the form of atmospheric emissions, liquid effluents and waste waters, sludges and solid wastes. The deposition of air pollution and the use of cadmium-contaminated water, sewage sludge and phosphate fertilizers cause the increase of the cadmium content in soils. Contamination of soils is particularly
troublesome because cadmium in soil is taken up efficiently by plants and thereby enters the food chain for humans and other animals.

1.1.3. Metabolism of cadmium in mammals

1.1.3.1. Absorption

Pulmonary absorption and gastrointestinal absorption are two major routes for cadmium uptake. Data from experimental animals and humans have shown that pulmonary absorption is higher than gastrointestinal absorption [26, 34, 35]. Depending on chemical speciation, particle size, and solubility in biological fluids, up to 50% of inhaled cadmium may be absorbed [36]. Absorption of ingested cadmium is influenced by the type of diet and nutritional status [26, 35, 37]. On average, 5% of the total oral intake of cadmium is absorbed, but individual values vary from less than 1% to more than 20% [2, 26, 35]. Absorption via skin is limited to only 0.2 - 0.8% of the applied dose [2].

1.1.3.2. Distribution

Distribution of cadmium varies according to the route of administration. In experimental animals, after a single administration of cadmium by the oral or parenteral routes, the highest concentration of cadmium is initially found in the liver [2]. However, the cadmium level in kidney increases for up to 8 months after exposure and then exceeds the level in the liver [38]. The pancreas and spleen also have relatively high concentrations of cadmium [39]. After repeated exposure, liver cadmium levels increase rapidly, then a redistribution of cadmium to the kidney occurs over a period of time [5]. The higher the intensity of exposure, the higher the initial ratio of liver-to-kidney concentration. The route of administration has been shown to be an important variable affecting the distribution of cadmium [40]. When cadmium is administered subcutaneously, 11 times more distributes in the liver than in the kidneys, whereas orally administered cadmium distributes equally between these two organs [40]. In humans, cadmium is stored principally in liver and kidneys where more than half of the body burden is deposited [26]. The highest
concentration is generally found in the renal cortex, whereas the lowest concentrations are found in the brain, bone, and fat [41, 42]. It has been calculated that about a third of the body burden of cadmium in a non-smoking male is in kidneys and almost a quarter in the liver and muscles [2]. These are the tissues in which cadmium has the longest biological half-time.

1.1.3.3. Excretion

The primary routes of cadmium excretion are in the urine and the feces. The average daily urinary cadmium excretion in mice, after subcutaneous injection, is about 0.01 - 0.02 % of the body burden [43]. Similar low urinary excretion rates have been found in rabbits and monkeys given oral or subcutaneous administration [44, 45]. However, as kidney dysfunction develops, a sharp increase in cadmium excretion occurs in mice, rabbits and rats, which leads to a decrease in renal and liver cadmium concentrations [44, 46, 47]. In humans, increased urinary cadmium excretion is also observed when tubular proteinuria develops [48]. It is very difficult to assess gastrointestinal excretion after oral exposure, since it is impossible to distinguish net gastrointestinal excretion from unabsorbed cadmium in faeces. However, both during and after parenteral exposure to cadmium, the total gastrointestinal cadmium excretion is considerably higher than the urinary excretion [43, 49]. After chronic exposure of rats, fecal excretion amounted to about 0.03 % of the body burden [49].

A number of studies have been carried out in order to assess the biological half-time of cadmium in experiment animals. Reported half-times range from several weeks in mice up to 22 years in monkeys [26, 45]. In humans, the biological half-time is estimated between 10 and 30 years [2].

1.1.3.4. Metallothionein

Metallothionein is a metal-binding protein of low molecular weight, which plays a key role in cadmium metabolism. It is unique in its high cysteine content and each molecule can bind seven
metal ions [50, 51]. Metallothionein is identified in human kidney and liver as well as in those of various experimental animals and is thought to play a role in cadmium transport and detoxification[52-54]. Synthesis of metallothionein can be induced by the essential metals zinc and copper, as well as by the toxic metals cadmium and mercury [55, 56]. After uptake of cadmium into blood plasma, it is usually bound to albumin which favors its distribution to the liver and various other organs [57]. In the liver, cadmium induces the synthesis of metallothionein and binds to it [58], forming a Cd-metallothionein complex. Then Cd-metallothionein appears in the blood plasma [57]. Because of its low molecular weight, Cd-metallothionein is quickly cleared by glomerular filtration and reabsorbed into the renal tubules [59]. Metallothionein is degraded in the lysosomes of the tubular cells, releasing cadmium into the cytosol, inducing metallothionein and binding to it, or damaging the sensitive sites of the cells in its ionic form [60].

1.1.4. Toxicology of cadmium

1.1.4.1. Acute toxicity

Acute toxic effects of cadmium on the gastrointestinal tract (after ingestion) and lung (after inhalation) were discovered soon after the isolation of cadmium in 1817 [61]. Acute poisoning from cadmium inhalation has been reported among workers shortly after exposure to fumes when cadmium metal or cadmium-containing materials are heated to high temperatures [62, 63]. The principal symptom is respiratory distress due to chemical pneumonitis and edema [61, 64]. In these cases, cadmium concentrations are usually very high. It is estimated that an eight hour exposure to 5 \( \text{mg/m}^3 \) cadmium may be lethal [26]. Acute food poisoning by cadmium occurred when cadmium was substituted for scarce chromium in the plating of many cooking utensils and containers during the period 1940 - 1950 [2]. Acute gastroenteritis occurred with the symptoms of severe nausea, vomiting, and abdominal pain. The absorption of cadmium was very limited due to the vomiting. The consequential short presence of cadmium in the gastrointestinal tract allowed for a rapid and complete recovery from acute poisoning [2].
1.1.4.2. Chronic toxicity

Long-term exposure to cadmium will cause chronic toxicity, predominantly in the lungs and kidneys [65]. The kidney is most frequently the critical target organ for cadmium. Renal dysfunction is one of the characteristic signs of chronic cadmium toxicity, which includes tubular dysfunction characterized by glycosuria, hypercalciuria, aminoaciduria, and low molecular weight proteinuria; and glomerular dysfunction manifested by decreased glomerular filtration and creatinine clearance, increased serum creatinine, and albuminuria [2, 61]. Numerous studies on renal damage in human following cadmium exposure have demonstrated that the renal changes are predominantly tubular [26, 65-67]. However, there are also some reports indicating that degenerative changes are evident even at the glomerular level [68, 69]. In a study on the influence of cadmium dust, the electrophoretic pattern of urinary proteins suggested that the lesion was first glomerular and later became predominantly tubular [48]. Pathological alterations show intense necrosis of the convoluted tubules, poor cell definition, and irregular granulations of the cells [61]. In rats given cadmium in their drinking water, the glomeruli show an apparent increase in mesangial matrix and basal lamina material, and the glomerular capillary lumina are reduced in size [70]. On the basis of available animal and human data, Friberg et al. [26] consider that it is justified to assume a value of 200 μg/g wet weight in the renal cortex as the critical cadmium concentration that can result in detectable renal dysfunction in human. In most cases cadmium-induced renal dysfunction is irreversible [2].

Chronic exposure to cadmium can also affect calcium metabolism and cause bone disease. The well-known Itai-itai disease which was prevalent in cadmium-polluted areas in Japan is characterized by osteomalacia and osteoporosis with a tendency to fracture accompanied by severe pain and renal dysfunction [5]. It has been estimated that the average daily oral intake of cadmium in this area was between 500 - 800 μg, whereas the average daily cadmium intake outside this area was around 30 - 50 μg [5]. Nogava et al. [71] suggested that cadmium-induced bone effects were mainly due to a disturbance in vitamin D and parathyroid hormone levels. Long-term inhalation of
cadmium causes chronic injury to the respiratory system, characterized by chronic obstructive airway disease [65].

1.1.5. Carcinogenicity of cadmium

Cadmium is regarded as a carcinogen in animals [4, 72]. It has also been classified as a category 1 carcinogen in humans by the International Agency for Research on Cancer (IARC) [3]. Inhaled cadmium compounds induce lung tumors in humans and experiments animals, and they also induce tumors of the prostate and testis in rats after subcutaneous injection and local tumors at various injection sites (im, ip and sc) [3]. The development of cancer is a multistage process. The process of tumor development requires the mutation of critical genes (initiation), stimulation of proliferation of committed cells (promotion), and transformation to malignant growth (progression). At high cytotoxic concentrations, cadmium induces DNA strand breaks and chromosomal aberrations which might be associated with tumor initiation [73, 74]. However, cadmium at nontoxic doses inhibits DNA repair and enhances the genotoxicity of directly acting mutagens [75, 76]. Since cadmium is only a weak mutagen [77, 78], the effective enhancement of endogenous mutations through inhibition of DNA repair may be a major mechanism reinforcing tumor initiation. Hartwig [79] reported the enhanced rate of mutation in response to a number of chemical mutagens and UV light in the presence of cadmium. At micromolar nongenotoxic doses, cadmium stimulates the expression of the proto-oncogenes c-fos, c-jun and c-myc, which is generally associated with tumor promotion and progression [12]. However, the mechanism by which cadmium induces the transcription of the proto-oncogenes remains unknown.

1.1.6. Cadmium and cellular signaling

Many investigations have focused on the molecular basis of cadmium toxicity and carcinogenicity. Cadmium has been reported to interfere with intracellular Ca\textsuperscript{2+} metabolism [7, 8], Zn\textsuperscript{2+} homeostasis [9], protein phosphorylation [10], and transcription factor modification [11]. Therefore, the molecular mechanism of cadmium toxicity and carcinogenicity may involve
interference with cellular signaling systems at different levels.

As already noted, cadmium has been observed to stimulate the expression of several immediate early response genes in different cell lines [13-15, 17]. These genes generally encode nuclear transcription factors that subsequently produce a number of gene products. For example, Fos and Jun heterodimers form the activating protein-1 (AP-1) transcription factor that binds to a consensus DNA sequence and leads to transcriptional activation of many genes [80]. When quiescent cells are stimulated to enter the cell cycle, expression of the proto-oncogenes is necessary for progression through $G_1$ and subsequent proliferation [81]. Therefore, cadmium appears to be a mitogenic stimulus in some cells.

Cadmium is also reported to induce apoptosis in a number of cells [17, 20-22]. Apoptosis, often referred to as programmed cell death, is a physiological process in which cells actively participate in their own destruction [82]. It is characterized by certain morphological features, including loss of membrane asymmetry and attachment, cell shrinkage, nuclear condensation, DNA fragmentation, and the formation of apoptotic bodies [83]. However, the effect of cadmium on apoptosis remains controversial. Although cadmium has been shown to cause apoptosis in a number of different cell lines [17, 20-22], it is also observed to block apoptosis induced by DNA-damaging metals such as chromium [84]. The dual effect of cadmium on apoptosis was also observed in previous work in our lab with rat mesangial cells (Z. Wang and D. M. Templeton, unpublished observations). After serum deprivation for several hours, in $Ca^{2+}$-replete medium, $Cd^{2+}$ protects against DNA fragmentation, whereas in low $Ca^{2+}$ medium, $Cd^{2+}$ enhances fragmentation. DNA damage can trigger apoptosis through a p53-mediated pathway. When DNA damage is detected, tumor suppressor protein p53 arrests the cell cycle in late $G_1$, and will initiate apoptosis if the damage is not repaired within a certain period of time [85, 86].

The tumor suppressor protein p53 is a 53 kDa transcription factor constitutively expressed in most cells. It binds DNA in a sequence-specific manner through a protein domain which is stabilized by the coordination of zinc within a tetrahedral cluster of three cysteine residues and one
histidine residue [87]. Recently, it has been reported that cadmium has two opposite effects on p53 protein [88]. At low concentrations, cadmium increases accumulation of p53 and its DNA binding activity with no increase in p53 mRNA. This effect is explained as a result of p53 protein stabilization by low levels of oxidative DNA damage induced by cadmium. However, at high concentrations, cadmium decreases p53 protein levels and DNA binding activity by metal substitution and conformational modifications that inactivate p53. Therefore, cadmium may either trigger apoptosis via the p53 pathway by causing DNA damage, inhibiting DNA repair, and increasing accumulation of p53 protein, or it may block apoptosis by inhibiting p53 DNA binding activity via conformational changes.

c-fos is generally regarded as an essential signal for cell proliferation [19]. However, a number of authors have also described c-fos as an important signal involved in apoptosis [23]. Because cadmium can be either a mitogenic or apoptotic stimulus, and has been found to induce c-fos in a number of cell lines [13, 15-18], elucidating the mechanism of c-fos induction by cadmium may help to explain the molecular mechanisms of cadmium toxicity and carcinogenicity and identify the role of c-fos in regulating cell destiny between life and death.

1.1.6.1. c-fos promoter elements

The c-fos proto-oncogene can be activated by a number of stimuli, including serum, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), interleukin-2 (IL-2), interleukin-6 (IL-6), phorbol ester TPA, Ca\(^{2+}\), cAMP, UV light, oxidants, and antioxidants [89]. These stimuli may target different regions on the c-fos promoter. Four proximal elements within the c-fos promoter have been identified as major targets for stimulating signals (Fig. 1): the sis-inducible element (SIE) [90, 91], the cAMP response element (CRE) [92], the serum response element (SRE) [92, 93], and the heat shock element (HSE) [94].

A) The sis-inducible element (SIE)

The SIE has been shown to mediate c-fos induction by conditioned medium from V-sis
Fig. 1. Regulatory elements within the human c-fos promoter. The specific locations of the four regulatory elements within the human c-fos promoter are shown and the examples of signals directed towards distinct regulatory elements are given [From 89].
transformed cells [90, 91]. It is involved in the induction of the c-fos gene by PDGF [90, 91] and EGF [95, 96], as well as by IL-2 [97] and IL-6 [98]. Inducible binding of the sis-inducible factor (SIF) to the SIE has been observed in vitro and in vivo [90, 99, 100]. The SIF includes three different complexes: SIF-A, SIF-B and SIF-C [95]. The SIF-A and SIF-B complexes, but not the SIF-C complex, can interact with the native SIE [98]. Further investigation indicates that two members of the signal transducer and activator of transcription (Stat) family constitute the SIF complex: SIF-A is a homodimer of Stat3, SIF-B is a heterodimer of Stat1α and Stat3, and SIF-C is a homodimer of Stat1α [95, 98, 101]. The Stats are activated and translocated to the nucleus in response to signals that activate the Janus kinase (Jak) group of tyrosine kinases [102]. After activation, the Stat3 homodimer or Stat1α/Stat3 heterodimer binds to the SIE and mediates SIE-dependent c-fos induction (Fig. 2).

B) The cAMP response element (CRE)

The CRE represents a binding site for CRE binding protein (CREB) and related transcription factors. It has been shown to be involved in transcriptional activation of c-fos in response to NGF [103], cAMP [104], Ca2+ and depolarization [89]. CREB contains a basic region leucine zipper motif that is responsible for dimerization and DNA binding [105]. Activation of CREB requires phosphorylation on Ser 133 [106], since only the phosphorylated form of CREB is able to interact with CREB binding protein (CBP), a coactivator which is required for the establishment of contact with the transcriptional machinery [107, 108]. CREB is a well known target of protein kinase A (PKA) which itself is activated by cAMP [109]. The PKA pathway is often initiated at the plasma membrane upon interaction of ligand with a G-protein-coupled serpentine receptor (Fig. 3). This induces the activation of adenylyl cyclase that converts ATP into cAMP [110]. cAMP interacts with the regulatory subunits of the inactive cytoplasmic form of PKA and subsequently results in the dissociation of the catalytic subunits of PKA, which then translocate to the nucleus, phosphorylate CREB on Ser 133 and thus activate CRE-dependent c-fos transcription [111]. Membrane depolarization leads to an influx of Ca2+ and results in CREB phosphorylation and CRE-mediated
Fig. 2. Signal transduction via the c-fos SIE. The mechanisms of c-fos induction by EGF and IL-2 via the c-fos SIE are shown. Only the homodimer of Stat3 and the heterodimer of Stat1α/Stat3, but not the homodimer of Stat1α, avidly bind with the c-fos SIE and subsequently lead to SIE-dependent c-fos induction [From 89].
Fig. 3. Signal transduction pathways targeting the c-fos CRE. Activation of CRE-dependent c-fos transcription requires phosphorylation of CREB on Ser 133. The possible mechanisms of c-fos induction by NGF, cAMP, Ca^{2+}, and depolarization are given [From 89].
c-fos induction in neuronal PC12 cells [89]. This process is thought to be mediated by the 
Ca²⁺/calmodulin-dependent protein kinase (CaMK) pathway (Fig. 3).

C) The serum response element (SRE)

The SRE was originally identified as mediating the transcriptional response of c-fos to serum [93]. Apart from serum, which is a complex mixture of different growth factors and other mitogens, a large variety of other stimuli also induce transcription activation of c-fos via the SRE, including individual growth factors as PDGF [112], EGF [113] and NGF [114], cytokines like IL-2 [115], intracellular messengers such as Ca²⁺ [113] and cAMP [116], phorbol ester TPA [112, 113], UV light [117], oxidants [118], and antioxidants [119]. The SRE is recognized by a dimer of the serum response factor (SRF) that recruits the monomeric ternary complex factors (TCFs) [120]. Dimerized SRF can autonomously interact with the SRE [121]. Several phosphorylation sites have been mapped within SRF and only the one at Ser 103 has been shown to be induced upon growth factor stimulation [122]. However, phosphorylation of SRF has so far not been shown to be involved in c-fos regulation, whereas phosphorylation of TCFs results in enhanced c-fos transcription [89]. Several different TCFs have been discovered, including Elk-1, Sap-1a/b, and Erp/Net [109]. These proteins form a subclass of the Ets family which is characterized by a DNA binding Ets domain [123]. TCFs are able to tightly bind certain Ets target sites but not the SRE that contains an Ets binding site [89]. Only additional protein-protein contact with SRE-bound SRF allows stable binding of TCFs to the SRE and subsequently induces c-fos [123].

Two major signaling systems are thought to be involved in SRE-dependent c-fos induction [109], the CaMK pathway and the MAPK cascades (Fig. 4). The CaMK pathway is initiated by membrane depolarization and the subsequent elevation of cytoplasmic Ca²⁺ levels [124]. Elevated cytoplasmic Ca²⁺ activates CaMK which in turn leads to SRF-linked transcription [122, 125]. The MAPK cascades participate in SRE-mediated c-fos induction through phosphorylating TCF at the C-terminal [89]. In mammalian cells, three subfamilies of the MAPK cascades have been identified,
Fig. 4. Signal transduction pathways involved in the c-fos SRE. Two major signaling systems involved in the c-fos SRE are shown. The CaMK pathway activates SRF, a member of the SRE ternary complex, whereas the MAPK cascades activate TCF, another member of the SRE ternary complex. The potential mechanisms of c-fos transcriptional activation by a number of different stimuli via the c-fos SRE are provided [From 109].
the extracellular signal-regulated kinases (ERK) pathway, the c-Jun-N-terminal/stress-activated protein kinases (JNK/SAPK) pathway, and the p38 pathway [109].

The ERK pathway can be triggered by serum, a mixture of different growth factors, and TPA, an activator of PKC (Fig. 5). When a growth factor binds to the cell surface receptor, it causes dimerization of growth factor receptor subunits and subsequent autophosphorylation on intracellular tyrosine residues [126]. The adapter molecule Grb2 interacts with the tyrosine-phosphorylated membrane receptor via its SH2 domain and recruits Sos to the plasma membrane via its SH3 domain [89]. Sos is a guanine nucleotide exchange factor [127]. After coupling with Grb2, it transforms the inactive GDP-Ras to the active GTP-Ras, which then recruits Raf to the plasma membrane leading to its activation [128]. Raf can also be activated by PKC in the absence of GTP-Ras [129]. This explains the induction of c-fos by TPA. Activated Raf triggers the signal transduction cascade of the ERK pathway [130]. Raf phosphorylates MEK1 and MEK2, which themselves are dual-specificity kinases phosphorylating a threonine and a tyrosine in the Thr-Glu-Tyr (TEY) activation motif of ERK [131]. ERK then enters the nucleus and phosphorylates Elk1 on its C-terminal [132]. Once activated, Elk1 in conjunction with SRF binds to the c-fos SRE and leads to transcriptional activation of c-fos [133].

The SAPK pathway can be initiated by cytokines and UV light (Fig. 4). Although a hierarchical cascade of the SAPK pathway has been identified [134], the molecular mechanism by which extracellular signals are transmitted from membrane receptors to the most upstream kinase of the SAPK pathway remains poorly understood. Rac and Cdc42, members of the Rho family of small GTPase, have been demonstrated to activate the SAPK pathway [135, 136]. Both of these G-proteins can activate a family of serine/threonine protein kinases called p21-activated kinases (PAKs) [137], which may start the SAPK pathway by activating MEKK1 [138], a kinase analogous to Raf in the ERK pathway. MEKK1 phosphorylates and activates SEK1 [139] and M KK7 [140], which further dually phosphorylate SAPK at the Thr-Pro-Tyr (TPY) motif [140-142]. Activated SAPK translocates into the nucleus, phosphorylates Elk1 and leads to SRE-mediated c-fos
Fig. 5. Activation of the ERK pathway. Cellular signaling via the ERK pathway from the cell surface to the nucleus is shown. Mechanisms of ERK activation by its typical stimuli are shown as well. Activated ERK subsequently translocates into nucleus, phosphorylates TCF and leads to the SRE-dependent c-fos induction [From 89].
induction [109].

The p38 pathway can be activated in response to hyperosmolarity (Fig. 4). However, less is known about the upstream components of this pathway compared with the above two pathways. Rac and Cdc42 have been confirmed to be upstream of p38, since constitutively active forms of both G-proteins have been shown to stimulate the activation of p38 [143, 144] whereas dominant negative Rac or Cdc42 inhibit p38 activity in response to IL-1 [143]. MKK3 and MKK6 are dual-specificity kinases that target the Thr-Gly-Tyr (TGY) activation motif of p38 [145]. Once activated, p38 may modulate transcription events by direct phosphorylation and activation of transcription factors. It is reported to phosphorylate and activate Elk1 in some cell lines while failing to do so in others [138, 146].

D) The heat shock element (HSE)

The HSE is responsive to heat shock, arsenite, heavy metals, and other stressors, such as ionizing radiation, toxic substances, hypoxia, oxidative stresses, inflammation, and viral infection [147-150]. These noxious stimuli turn on the stress response, also termed heat shock response, which is mediated by the HSE, leading to increased expression of the HSE-bearing genes, including stress proteins, also called heat shock proteins (HSP) [147]. These stimuli are also known to induce c-fos in some cell lines [94, 151]. Although it was generally thought that the HSE was absent from the c-fos promoter and that c-fos induced by stressors is mediated by a mechanism other than HSE-mediated transcription [152], it has been found recently that an HSE exists in the human and rodent c-fos promoter and is highly responsive to heat, arsenite, and cadmium in a number of cells [94]. Therefore, the HSE appears to be another candidate to regulate c-fos expression in response to various physiological and pathological stimuli.

It should be noted that most stimuli targeting the SIE such as EGF, PDGF, and IL-2 have been shown to also stimulate the SRE [112, 113, 115]. Similarly, NGF, Ca^{2+} and cAMP not only activate the CRE but also the SRE [113, 114, 116]. In addition, stressors such as heat, arsenite, and
cadmium which are found to enhance the HSE activity may also stimulate the SRE [152]. It seems that signaling to the c-fos promoter may always involve the SRE (Fig. 1). The SRE appears to be the pivotal regulatory sequence in the c-fos promoter.

1.2. Biology of Mesangial Cells

1.2.1. Glomerulus

The functional unit of the kidney is the nephron. Each nephron is composed of the glomerulus, the Bowman’s capsule, the proximal tubule, the loop of Henle, the distal tubule and the collecting duct. The glomerulus is a tuft of capillaries supported by a mesangial network and surrounded by Bowman's capsule (Fig. 6). At the vascular pole, the afferent arteriole enters, branching into four to eight lobules to form the capillary tuft. The capillaries converge into the efferent arteriole which leaves the glomerulus at the vascular pole. The mesangial cells, together with their surrounding matrix material localize within the axes of the individual lobules. Endothelial cells line the lumen of the capillaries and the visceral epithelial cells cover the capillaries from the outside. The glomerular basement membrane (GBM) separates the epithelium from the endothelium or the mesangium. At the vascular pole, the visceral epithelium and the GBM are reflected into the parietal epithelium, forming Bowman’s space, which collects plasma filtrate. The parietal epithelium of Bowman’s capsule passes over into the epithelium of the proximal tubule at the urinary pole. The major function of the glomerulus is filtration. The fenestrated endothelial cells, the GBM, the visceral epithelial cells, and inter-epithelial slit-pores together form the filtration barrier, which selectively filters plasma proteins according to their size and charge [153].

1.2.2. Mesangial cells

The mesangial cells, together with the surrounding matrix material, form the mesangium. The cells are quite irregular in shape and occupy the central region of the glomerular tuft. They have an indented nucleus, a small amount of cytoplasm, and relatively few and poorly developed ribosomes, endoplasmic reticulum, and stacked Golgi cisternae [154, 155]. The mesangial cells
Fig. 6. Diagram of a glomerulus in longitudinal section. At the vascular pole, the afferent arteriole (AA) enters and the efferent arteriole (EA) leaves the glomerulus. The glomerular capillaries are outlined by fenestrated endothelia (E) and covered from the outside by the visceral epithelia termed podocytes (PO). The glomerular basement membrane (GBM) is continuous throughout the glomerulus. The mesangial cells (M) occupy the axial region of the glomerular lobules. At the urinary pole, the parietal epithelia (PE) of Bowman's capsule pass over into the epithelia of the proximal tubule (P) and the urinary space (US) passes over into the tubule lumen [From 153].
exhibit certain properties of smooth muscle cells, indicating their same origin. A prominent feature is the contractile phenotype which is characterized by the presence of numerous bundles of small intracellular fibrils and associated attachment bodies similar to those found in the smooth muscle cells [155-157]. The similarity between these two cell types is further demonstrated by the presence of receptors for angiotensin II, endothelin, and arginine vasopressin on the plasma membrane of the mesangial cells [158, 159]. The contractility of the mesangial cells plays an important role in the regulation of glomerular hemodynamics. Contraction changes glomerular capillary diameter by pulling the GBM together from opposing mesangial angles and subsequently assists afferent and efferent arterioles in regulating glomerular plasma flow and filtration rate [159]. Another important function of the mesangial cells is to synthesize extracellular matrix components which combine in an organized manner to make up the mesangial matrix and provide structural support to the glomerulus. Mesangial cells are reported to synthesize collagen type I, III, IV and V [160], fibronectin [161], laminin [161], and chondroitin and heparan sulfate proteoglycans [162] in culture. In addition, they also synthesize the different classes of proteinases, IL-1, IGF-1, and PDGF [163].

In normal adult kidney, the mesangial cells are mostly quiescent with an extremely low renewal rate [164]. Proliferation of mesangial cells represents a condition where the equilibrium between proliferation and apoptosis is not balanced [165]. This process can be triggered by growth factors such as PDGF, IGF-1, or bFGF, and other pathological stimuli [164, 166]. Much evidence confirms that proliferation of mesangial cells is a major feature of glomerular injury [167-169]. Numerous glomerular diseases, including lupus nephritis, IgA nephropathy, and variants of idiopathic focal glomerulosclerosis are characterized by proliferation of cells in mesangial regions [170, 171]. Activation of a synthetic phenotype of mesangial cells is another feature of glomerular injury which is characterized by well-developed rough endoplasmic reticulum and Golgi apparatus, an increase of extracellular matrix synthesis and secretion, up-regulation of expression of SMC-like protein (α-SM actin), and secretion of interstitial collagens which are not normally present in the mesangial matrix [172-174]. The expansion of the mesangial matrix ultimately results in focal
glomerulosclerosis [170, 175]. The secretion of cytokines and growth factors by mesangial cells may influence proliferation of all the cells in glomerulus.

Cadmium has been observed to be taken up by renal mesangial cells [176], and cause greater toxicity to mesangial cells than to the other cells in the isolated glomerulus [177]. Its ionic form is much more toxic to mesangial cells than Cd-metallothionein [178]. These observations indicate mesangial cells to be another target for cadmium renal toxicity in addition to proximal tubular cells. Furthermore, Cd^{2+} has been found to induce c-fos transcription in mesangial cells [18]. Therefore, we will further use mesangial cells as our cell model to investigate the mechanism of c-fos induction by cadmium.

1.3 Hypothesis and objective

In this thesis, we will study the molecular basis of c-fos induction by cadmium. As mentioned above, a large variety of extracellular stimuli induce c-fos transcription by targeting the SRE region of the c-fos promoter. Thus, we will focus on the intracellular signaling involved in this element. As one of two major signaling systems involved in SRE-dependent c-fos induction, the CaMK pathway can mediate c-fos induction by phosphorylating and activating the SRF [122, 125]. It appears to be a candidate to mediate c-fos induction by cadmium. However, previous work in our lab demonstrated that Cd^{2+} had no effect on either autonomous or total CaMKII activity in mesangial cells, and neither the general CaMK inhibitor KT5926, nor the CaMKII-specific inhibitor KN93 caused any significant diminution in response of c-fos mRNA to Cd^{2+} [18], indicating the CaMK pathway is not involved in induction of c-fos by cadmium. Therefore, we will further investigate other signaling systems involved in SRE-dependent c-fos induction, namely, the MAPK cascades. Since several members of the MAPK cascades have been shown to phosphorylate and activate Elk1 [109], which forms part of the transcription factor complex that binds to the SRE, we hypothesize that induction of c-fos by cadmium in mesangial cells is mediated by the MAPK cascades. Our objective is to identify which subfamilies of the MAPK cascades are activated by cadmium and subsequently mediate c-fos induction.
2. MATERIALS AND METHODS

2.1. Reagents and Materials

Reagents for cell culture, lipofectamine 2000 reagent and plasmid pCMV-SPORT-βgal for cell transfection, TRIzol reagent for RNA isolation, and M-MLV reverse transcriptase and primers for RT-PCR were obtained from Gibco/BRL (Burlington, Ont., Canada). 12-O-tetradecanoylphorbol-13-acetate (TPA) was from R & D Systems (Minneapolis, MN, U.S.A.). The MEK1/2 inhibitor PD98059 was from New England Biolabs (Mississauga, Ont., Canada). Myelin basic protein and protease inhibitors aprotinin, leupeptin and pepstatin were products of Sigma (St. Louis, MO, U.S.A.). A random primed DNA labeling kit was from Boehringer Mannheim (Laval, Que., Canada). Protein A-Sepharose, EcoR I, DNase I, RNase inhibitor, and Taq polymerase were from Pharmacia (Uppsala, Sweden). GST-c-jun fusion protein, anti-rabbit IgG-HRP, anti-goat IgG-HRP, goat polyclonal anti-FLAG antibody, and rabbit polyclonal anti-HA, anti-ERK2 and anti-JNK1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). ECL Western blotting analysis system was ordered from Amersham Canada (Oakville, Ont., Canada). [α-32P]dCTP (specific radioactivity 3000 Ci/mmol) and [γ-32P]ATP (specific radioactivity 4500 Ci/mmol) were from NEN Life Science Products (Boston, MA, U.S.A.) and ICN (Costa Mesa, CA, U.S.A.). A rat c-fos cDNA cloned by T. Curran [179] was obtained from T. Cruz (University of Toronto) and a cDNA for mouse 18S rRNA was obtained from J. Koropatnick (University of Western Ontario). pcDNA3.1/Zeo(+), HA-SEK1-AL pcDNA3.1/Zeo(+), and FLAG-MKK7-K76E pCR3.1 were generous gifts from J. P. Woodgett (University of Toronto). The primers for SEK1-AL (forward 5'TACGATGTCCAGATTACGC; reverse 5'CCATGAGAAGTTGTTTTTGT) and the primers for M KK7-K76E (forward 5'CTACAGGACGACGTGA; reverse 5'CAAGGCCGCCACTGATGC) were ordered from Gibco/BRL (Burlington, Ont., Canada).
2.2. Cell culture

Rat mesangial cells (RMC) were isolated from glomeruli of kidneys of male Wistar rats and identified by their characteristic morphology, cytoskeletal histochemistry, and contractile properties as previously described [180, 181]. They were grown in RPMI 1640 medium with penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37°C. The cells were passaged by trypsinization and used between the fifth and fifteenth passage for all experiments. Quiescence was induced by starving cultures at 60-80% confluence with medium containing 0.4% FBS for 48 h. Quiescent cells were then treated with different stimuli under different conditions.

2.3. Cell transfection

Rat mesangial cells were grown in 10 cm Petri dishes. When cells were 80-90% confluent, the medium was changed with 15 ml DMEM medium containing 10% FBS. 3 ml transfection solution with 30 μg plasmid and 90 μl lipofectamine 2000 reagent was added directly into each dish and incubated at 37°C for 5 h. The medium was replaced with 10 ml RPMI 1640 medium containing 10% FBS and incubated at 37°C overnight followed by 48 h starvation in 10 ml RPMI 1640 medium with 0.4% FBS. The transfected cells were then exposed to different stimuli according to the design of each experiment.

2.4. Transfection efficiency study

Transfection efficiency was determined by *in situ* β-galactosidase staining of transfected cells [182]. Rat mesangial cells were transfected with pCMV-SPORT-βgal in 24-well plates. After transfection for 24 h, cells were washed once with 2 ml phosphate-buffered saline (PBS), followed by 5 min fixation at room temperature in 1 ml of fixative containing 2.7 mM KCl, 1.1 mM KH₂PO₄, 140 mM NaCl, 8.1 mM Na₂HPO₄·7H₂O, 2% formaldehyde, and 0.05% glutaraldehyde. Cells were washed twice with 2 ml PBS and then incubated with 1 ml of substrate/stain solution (2.7 mM KCl, 1.1 mM KH₂PO₄, 140 mM NaCl, 8.1 mM
Na₂HPO₄·7H₂O, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, and 0.1 % X-gal) overnight at 37°C. After rinsing with 2 ml PBS, cells were observed under a microscope and photographed.

2.5. Cell viability study

Cell viability was assessed by the tetrazolium salt (MTT) assay [183]. Rat mesangial cells were cultured in RPMI 1640 medium in 24-well plates. At 60 - 80 % confluence, cells were starved with medium containing 0.4 % FBS for 48 h. After quiescence was induced, cells were exposed to 10 μM CdCl₂ for different periods of time from 5 min to 24 h. Cells were then washed with PBS and incubated with 1 mg/ml MTT in 100 μl of RPMI 1640 at 37°C for 1 h. After washing the medium, cells were treated with 420 μl DMSO and shaken at room temperature for 30 min. A 100 μl sample from each well was transfer to a 96-well plate and the optical density (OD) at 570 nm for each sample was obtained in a DuPont mutiwell spectrophotometer.

2.6. RNA isolation and Northern blot analysis

Total RNA was isolated using TRIzol Reagent, as described by Chomczynski and Machey [184]. Equal amounts of RNA (10 - 20 μg) were denatured by the method of Gong [185], separated by electrophoresis on agarose-formaldehyde gels (1.2 % agarose, 2.2 M formaldehyde, 5 mM sodium acetate, 1 mM EDTA, 20 mM MOPS, pH 7.0), and transferred to Hybond-N nylon membrane by overnight capillary blotting. Blots were pre-hybridized at 42°C for 4 - 6 h in pre-hybridization solution containing 10 % dextran sulfate, 5 x SSPE (900 mM NaCl, 50 mM Na₂HPO₄, 50 mM NaH₂PO₄, 0.5 mM EDTA), 50 % formamide, 5 x Denhardt's solution, 250 μg/ml salmon sperm DNA, and 0.5 % SDS. Hybridization was then performed by adding c-fos cDNA which was labeled with [α-³²P]dCTP by the random primer method into prehybridization solution and incubated at 42°C overnight. Levels of c-fos mRNA were quantitated by densitometry of the Northern blot autoradiograph and normalized to 18S rRNA after probing with labeled cDNA to rat 18S rRNA.
2.7. Kinase assays

2.7.1. ERK assay

Cells were washed twice with ice-cold PBS and scraped into 800 μl of lysis buffer [50 mM Tris-HCl, pH 7.4, 1 % (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EGTA] containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and phosphatase inhibitors (1 mM Na₃VO₄, 1 mM NaF). After sonication twice for 5 s and centrifugation at 10,000 x g for 15 min, cytosolic protein content was determined by the method of Peterson [186]. Aliquots of cytosol containing 400 μg of protein were precleared by adding 1 μg of normal rabbit IgG with 20 μl of protein A-Sepharose and centrifuging in a microcentrifuge for 5 min. The supernatant was incubated with 2 μg of anti-ERK2 antibody for 3 h at 4°C, and immunoprecipitates were recovered by incubation with a 50 % slurry of protein A-Sepharose for a further 2 h. ERK activity in the immunoprecipitates was determined by phosphorylation of myelin basic protein (MBP). Immunoprecipitates were mixed with 20 mM HEPES buffer, pH 7.0, 10 mM MgCl₂, 2 mM MnCl₂, 0.5 mM EGTA, 10 mM NaF, 0.5 mM Na₃VO₄, 1 mM dithiothreitol (DTT), 0.5 mg/ml MBP, 50 μM ATP and 5 μCi of [γ-³²P]ATP, and incubated at 30°C for 20 min. Reaction was stopped by adding 1/4 volume of 5 x SDS sample buffer (250 mM Tris-HCl, pH 6.8, 500 mM DTT, 10 % SDS, 0.5 % bromophenol blue, and 50 % glycerol) and subjected to 14/7 % SDS-polyacrylamide gel electrophoresis according to Laemmlli [187]. Incorporation of ³²P into MBP was determined by autoradiography and densitometry.

2.7.2. SAPK assay

Cells were lysed in 800 ml of lysis buffer (25 mM HEPES, pH 7.5, 1 % Triton X-100, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20 mM β-glycerophosphate, 0.1 % SDS, 0.5 % sodium deoxycholate, 0.5 mM DTT, 0.1 mM Na₃VO₄, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cytosolic protein was collected as described above. 1 μg normal
rabbit IgG was added to 400 μg cytosolic protein followed by 20 μl of protein A-Sepharose. The supernatants were then immunoprecipitated with anti-JNK1 antibody for 3 h followed by incubation with 50 % protein A-Sepharose slurry. Washed immunoprecipitates were incubated with 30 μl of kinase assay buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM NaF, 3.3 mM DTT, 0.5 mM Na3VO4, 20 μM ATP, 2 μg GST-c-jun fusion peptide, and 1 μCl [γ-32p]ATP) at 30°C for 30 min. The reaction was terminated by adding 1/4 volume of 5 x SDS sample buffer. The samples were separated by 14/7 % SDS-polyacrylamide gel electrophoresis followed by autoradiography and densitometry.

2.8. Immunoprecipitation and Western blot analysis

Cell lysates containing 400 μg of protein were immunoprecipitated with 2 μg anti-HA antibody or anti-FLAG antibody. The immunoprecipitates were recovered with protein A-Sepharose, resolved by by 14/7 % SDS-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membrane via electrophoretic transfer. The blots were then probed with anti-HA antibody or anti-FLAG antibody followed by horseradish peroxidase-labeled secondary antibodies and developed by using the enhanced chemiluminescence (ECL) detection system.

2.9. RT-PCR

2.9.1. First strand cDNA synthesis

Total RNA was used for reverse transcription-PCR (RT-PCR). To prevent contamination of the RNA with DNA, 2 μg total RNA was mixed with 1 μl DNase I (7.5 U/μl) and 1 μl 10 x DNase I reaction buffer (400 mM Tris-HCl, pH 7.5, 60 mM MgCl2) in a total volume of 10 μl, and incubated at 37°C for 10 min. The reaction was terminated by adding 1 μl 20 mM EDTA and heating to 65°C for 15 min. Reverse transcription was then performed in 10 μl of reaction mixture containing 2 μl DNase I reaction mixture, 1 μl RNase inhibitor (32 U/μl), 1 μl 10 mM dNTP, 1 μl 10 x Hexanucleotide Mixture (500 mM Tris-HCl, pH 7.2, 100 mM MgCl2, 1 mM DTE, 2 mg/ml BSA, 62.5 U/ml random primer), 1 μl M-MLV reverse transcriptase (200 U/μl), and incubated at
37°C for 1 h.

2.9.2. PCR amplification

PCR was carried out by mixing 2.5 μl first strand cDNA, 2.5 μl 10 μM of each corresponding primer, 0.75 μl 10 mM dNTP, 2.5 μl 10 x PCR buffer (100 mM Tris-HCl, pH 9.0, 15 mM MgCl₂, 500 mM KCl), 0.3 μl Taq polymerase (5 U/μl) and 14.25 μl water into a total volume of 25 μl. The reaction mixture was first incubated at 94°C for 5 min, followed by 45 cycles of reaction (94°C for 30 sec; 55°C for 45 sec; 72°C for 30 sec) and terminated by a final incubation at 72°C for 5 min. The RT-PCR products were applied to 1% agarose gel. Electrophoresis was performed at 100 V for 1 h followed by photographing the gel under UV light.

2.10. Statistics

Statistical significance was determined either by one sample Student's t-test when comparing to a 100% control value, or by unpaired Student's t-test for all the experiments, using StatWorks™ software.
3. RESULTS

3.1. Effects of Cd\(^{2+}\) on c-fos expression

When rat mesangial cells are serum-starved for 48 h, they become quiescent. Serum starvation arrests the growth of mesangial cells and causes them to revert to G\(_0\) [188, 189]. When quiescent mesangial cells are stimulated with serum or the phorbol ester, TPA, they re-enter the cell cycle and induce the expression of c-fos mRNA within 15 - 30 min [190]. Our lab previously showed that in quiescent rat mesangial cells, 1 \(\mu\)M Cd\(^{2+}\) caused a transient increase in c-fos mRNA which appeared in 30 min and then decreased, whereas 10 \(\mu\)M Cd\(^{2+}\) stimulated a different pattern of c-fos expression which presented in 30 min, plateaued at 2 h and remained elevated for at least 8 h [18]. To further investigate this phenomenon in detail, the 24 h time course of c-fos expression in response to Cd\(^{2+}\) was measured at each concentration. Both concentrations induced a similar significant increase of c-fos mRNA to about three times the basal level at 30 min which declined thereafter (Fig. 7, \(p < 0.05\)). However, only 10 \(\mu\)M Cd\(^{2+}\) caused a second peak nearly seven-fold the basal level at 8 h that then declined. Since treatment with each concentration of Cd\(^{2+}\) in this protocol involved a medium change at time zero, we wondered if the components in the medium would interfere with the Cd\(^{2+}\) treatment and cause the initial phase of increase in c-fos mRNA. Therefore, we also tested the 24 h time course of a medium change alone. At 30 min, c-fos mRNA did not show any significant increase compared with time zero (Fig. 7).

Since a biphasic increase of c-fos mRNA was only observed by treatment with 10 \(\mu\)M Cd\(^{2+}\), whereas the initial phase appeared to be similar between 1 \(\mu\)M and 10 \(\mu\)M Cd\(^{2+}\), we further checked the concentration dependent c-fos expression in response to Cd\(^{2+}\) at 30 min and 8 h. At the earlier time, even 0.1 \(\mu\)M Cd\(^{2+}\), the lowest concentration tested, caused an approximate 50 % increase in c-fos mRNA which remained similar at higher concentrations up to 3 \(\mu\)M, while 10 \(\mu\)M Cd\(^{2+}\) caused a further increase in c-fos mRNA (Fig. 8). At the later time, 3 \(\mu\)M Cd\(^{2+}\) was the lowest concentration to be observed to significantly increase c-fos mRNA (\(p < 0.05\)). Therefore, a
Fig. 7. Time course of c-fos induction by Cd\textsuperscript{2+}. Quiescent mesangial cells were treated with either 1 μM (black bars) or 10 μM (white bars) CdCl\textsubscript{2} for the indicated times in serum-free medium. Total RNA was isolated and analyzed by Northern blotting for c-fos mRNA and corrected for loading by 18S rRNA. The bars show the mean ± s.e. from three independent experiments at each concentration. The gray bars show the effect of a medium change alone, without added Cd\textsuperscript{2+} (n = 4). Values are expressed relative to the basal level at time zero taken as 100 %. Significant increases above the basal level were determined by Student's t test; a - p < 0.05, b - p < 0.005, c - p < 0.001.
Fig. 8. Dose dependence of early and late induction of c-fos by Cd^{2+}. Quiescent mesangial cells were treated with the indicated concentration of CdCl₂ and total RNA collected for Northern blotting of c-fos mRNA at either 30 min or 8 h. A representative Northern blot probed for c-fos and 18S RNA is shown in the top panel. Signals were quantitated by densitometry, corrected for 18S rRNA, and expressed as a percentage of the basal level present at 30 min in untreated cells. Values are mean ± s.e. from four independent experiments. * Denotes significantly greater than basal signal at p < 0.05.
concentration as low as 0.1 $\mu$M Cd\textsuperscript{2+} was enough to cause the initial phase increase in c-fos mRNA, whereas over 3 $\mu$M was required to induce the sustained second phase.

3.2. Involvement of the ERK pathway in c-fos induction by Cd\textsuperscript{2+}

3.2.1. Effect of Cd\textsuperscript{2+} on ERK activation

As a member of the MAPK family, ERK can phosphorylate and activate Elk1 [89], which further binds to SRE-bound SRF dimer and causes SRE-dependent c-fos induction. The ERK pathway appears to be a candidate to mediate c-fos induction by cadmium. To assess the involvement of the ERK pathway in Cd\textsuperscript{2+}-dependent c-fos induction, we examined the time course of 10 $\mu$M Cd\textsuperscript{2+} on ERK activity. ERK activity showed a biphasic increase in response to 10 $\mu$M Cd\textsuperscript{2+} with the first peak at 15 min and the second peak at 8 h (Fig. 9), similar to the time course of c-fos mRNA in response to Cd\textsuperscript{2+}. The dose dependence of ERK activation in response to Cd\textsuperscript{2+} was tested at 15 min and 8 h. At 15 min, different concentrations of Cd\textsuperscript{2+} produced a similar modest increase in ERK activity that nevertheless did not reach statistical significance (Fig. 10). However, at 8 h, only 10 $\mu$M Cd\textsuperscript{2+} caused a significant increase in ERK activity ($p < 0.05$). ERK showed the similar patterns of time course and concentration-dependent activation in response to Cd\textsuperscript{2+} as c-fos mRNA, suggesting its involvement in c-fos induction by Cd\textsuperscript{2+}.

3.2.2. Effect of PD98059 on ERK activation

To further assess the role of ERK activation by Cd\textsuperscript{2+} in Cd\textsuperscript{2+}-dependent c-fos induction, we employed PD98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one]. PD98059 is a synthetic chemical that selectively inhibits the ERK-activating kinase, MEK1/2, without significant inhibitory effect on ERK itself (Fig. 11) [191]. It inhibits both the activation and phosphorylation of MEK1/2 through an allosteric mechanism [191]. Its IC\textsubscript{50} is 10 $\mu$M for MEK1 and 50 $\mu$M for MEK2. PD98059 is a highly selective inhibitor for the ERK pathway without inhibitory effect on either the SAPK or the p38 pathways. We examined the effect of PD98059 on ERK activation
Fig. 9. Time course of ERK activation by 10 μM Cd^{2+}. Quiescent mesangial cells were treated with 10 μM CdCl₂ for different times as indicated. Cell extracts were immunoprecipitated with anti-ERK2 antibody. ERK activity was measured by the ability of the immunoprecipitates to incorporate [γ-^{32}P] into the substrate MBP. Incorporation of ^{32}P into MBP was measured by autoradiography and densitometry. The autoradiogram is from one representative experiment. All time points are significantly elevated (p < 0.05) above the time zero point taken as 100 %. Values are mean ± s.e. from three separate experiments.
Fig. 10. Dose dependence of activation of ERK by Cd^{2+}. Quiescent mesangial cells were treated with the indicated concentration of CdCl_2 and either 15 min or 8 h later (as indicated by the horizontal bars) cell extracts were collected for measurement of ERK activity. The signal at 15 min after a medium change in the absence of Cd^{2+} is taken as 100% and values are expressed relative to this basal level. Values are mean ± s.e. from four separate experiments. * Denotes significantly higher than basal level (p < 0.05).
Fig. 11. **PD98059 blocks the ERK pathway.** PD98059 selectively inhibits the ERK pathway by preventing activation and phosphorylation of MEK1/2.
stimulated by Cd\(^{2+}\). Since TPA is a well established activator of the ERK pathway through activating PKC, we examined the effect of PD98059 on TPA-dependent ERK activation as well. Both Cd\(^{2+}\) and TPA caused marked increases in ERK activity (Fig. 12). However, at 50 μM, PD98059 significantly decreased ERK activity stimulated by TPA and completely blocked ERK activity stimulated by Cd\(^{2+}\).

3.2.3. Effect of PD98059 on c-fos induction

The above data showed that PD98059 can prevent Cd\(^{2+}\)-dependent ERK activation. To confirm the involvement of Cd\(^{2+}\)-dependent ERK activation in Cd\(^{2+}\)-dependent c-fos induction, we need to provide further evidence that blocking ERK activation will attenuate c-fos mRNA induced by Cd\(^{2+}\). Therefore, we checked the dose-dependent effect of PD98059 on c-fos induction by Cd\(^{2+}\). Since TPA is well known to induce c-fos through the ERK pathway, we also observed the dose-dependent effect of PD98059 on TPA-dependent c-fos induction. PD98059 decreased the c-fos mRNA induced by 4 h exposure to 10 μM Cd\(^{2+}\) (Table 1). However, the decrease did not show a dose-dependence. Even at 100 μM, which blocked Cd\(^{2+}\)-dependent ERK activation completely, the inhibitor only caused a 24% decrease in c-fos mRNA induced by 10 μM Cd\(^{2+}\). In contrast, PD98059 showed a dose-dependent inhibitory effect on TPA-dependent c-fos induction, and at 100 μM, the inhibitor suppressed TPA-induced c-fos mRNA to the basal level (Table 1). Blocking the ERK pathway with PD98059 partially inhibited Cd\(^{2+}\)-dependent c-fos induction, indicating the involvement of the ERK pathway in mediating c-fos induction by Cd\(^{2+}\), but also suggesting the participation of additional mechanisms in this process.

3.3. Involvement of the SAPK pathway in c-fos induction by Cd\(^{2+}\)

3.3.1. Effect of Cd\(^{2+}\) on SAPK activation

As one of the stressors in mammalian cells, cadmium has a potential to activate the SAPK pathway. Furthermore, SAPK can phosphorylate Elk1 on its C-terminal and subsequently lead to
Fig. 12. Effect of PD98059 on ERK activation. Quiescent mesangial cells were stimulated with either 10 μM CdCl₂ (solid bars) for 15 min or 50 ng/ml TPA (open bars) for 5 min in the presence or absence of 50 μM PD98059. Cell extracts were immunoprecipitated with anti-ERK2 antibody for measurement of ERK activity. Values are expressed relative to basal activity taken as 100 % within each set of treatments (left hand axis for Cd²⁺, right hand axis for TPA). Values are mean ± s.e. from three independent experiments.
Table 1. Effect of PD98059 on c-fos induction. Quiescent mesangial cells were pretreated with different concentrations of PD98059 for 1 h prior to exposure either to 10 μM CdCl₂ for 4 h or 50 ng/ml TPA for 30 min. Levels of c-fos mRNA were quantitated by densitometry of the Northern blot autoradiograph and normalized to 18S rRNA. Values are expressed as a percentage of the maximum level achieved in the absence of inhibitor taken as 100%. Basal levels prior to addition of CdCl₂ or TPA were, respectively, 7% and 17% of maximum.

<table>
<thead>
<tr>
<th>[PD98059] (μM)</th>
<th>c-fos mRNA/18S rRNA (%)</th>
<th>c-fos mRNA/18S rRNA (%)</th>
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<tbody>
<tr>
<td></td>
<td>10 μM Cd²⁺</td>
<td>50 ng/ml TPA</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>84.9</td>
<td>88.0</td>
</tr>
<tr>
<td>10</td>
<td>86.1</td>
<td>75.1</td>
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<tr>
<td>50</td>
<td>85.9</td>
<td>61.7</td>
</tr>
<tr>
<td>100</td>
<td>76.2</td>
<td>17.4</td>
</tr>
</tbody>
</table>
SRE-dependent c-fos induction [109]. The SAPK pathway appears to be an alternative pathway that may be involved in mediating c-fos induction by Cd2+. Thus, we examined the effects of Cd2+ on SAPK activity. SAPK showed biphasic activation in response to 10 μM Cd2+ with the early peak at 15 min and the late peak at 8h (Fig. 13), consistent with the biphasic induction of c-fos in response to Cd2+. However, because of greater between-experiment variability in this assay, the increments only reached statistical significance at 1 h, 16h and 24h (p < 0.05).

We further observed the effects of different concentrations of Cd2+ on SAPK activity at 15 min and 8 h. Different concentrations of Cd2+ caused similar 50 % increases in initial SAPK activity, whereas concentrations at 3 μM or higher were required to stimulate sustained SAPK activation (Fig. 14). The similar patterns of time course and concentration-dependent response to Cd2+ between SAPK activity and c-fos mRNA indicate the SAPK pathway to be another candidate involved in Cd2+-dependent c-fos induction.

3.3.2. Effects of kinase-inactive mutants of SEK1 and MKK7 on SAPK activation

To confirm that Cd2+-dependent SAPK activation is involved in c-fos induction by Cd2+, we planned to inhibit the SAPK pathway and expected to see the subsequent decrement in c-fos mRNA induced by Cd2+. Recently, two upstream kinases of SAPK have been identified, SEK1 [139, 141] and MKK7 [140, 192]. It is reported that SEK1 activates both SAPK and p38 [193], whereas MKK7 is a specific activator for SAPK [192]. Since no synthetic inhibitors for these two kinases are available, we tried to block the SAPK pathway by transfecting the rat mesangial cells with kinase-inactive mutants of either SEK1 or MKK7. A kinase-inactive mutant of SEK1 was designed by mutating two potential phosphorylation sites from Ser 220 to Ala and Thr 224 to Leu [139] and inserted into the vector pcDNA3.1/Zeo(+) after tagging with HA at the 5' end. A kinase-inactive mutant of MKK7 was created by substituting Lys 76 in the ATP binding domain with Glu [140] and cloned into the vector pCR3.1 by PCR with the first primer adding a FLAG epitope at the 5' end. The vector pcDNA3.1/Zeo(+) was taken as an empty vector control. The three plasmids,
Fig. 13. Time course of SAPK activation by 10 μM Cd^{2+}. Quiescent mesangial cells were treated with 10 μM CdCl₂ for the indicated times. Cell extracts were immunoprecipitated with anti-JNK1 antibody and assayed for SAPK activity with GST-c-Jun as the substrate. Incorporation of [γ-^{32}P] ATP into GST-c-Jun was detected by autoradiography and quantitated by densitometry. A representative autoradiogram was provided on the top. Values are mean ± s.e. from three independent experiments and are expressed relative to the basal activity at time zero taken as 100%. * Denotes a significant elevation above the basal level (p < 0.01).
Fig. 14. Dose dependence of activation of SAPK by Cd\(^{2+}\). Quiescent mesangial cells were treated with the indicated concentration of CdCl\(_2\) and either 15 min or 8 h later (as indicated by the horizontal bars) cell extracts were prepared for measurement of SAPK activity. Values are mean ± s.e. from three independent experiments and are expressed relative to the basal activity at 15 min after a medium change without added Cd\(^{2+}\) taken as 100 %. Significant increases above basal level are indicated by (a) p < 0.01 or (b) p < 0.05.
pcDNA3.1/Zeo(+), HA-SEK1-AL pcDNA3.1/Zeo(+), and FLAG-MKK7-K76E pCR3.1, contain 5015 bp, 6195 bp, and 6375 bp respectively (Table 2).

We first did preliminary experiments to find out the optimal conditions for transfecting rat mesangial cells. By using the method of in situ β-galactosidase staining [182], we found that under the optimal conditions the transfection efficiency in rat mesangial cells could reach around 40 % (Fig. 15). Then we transfected rat mesangial cells with either vector, or SEK1-AL, or MKK7-K76E. Since SEK1-AL was tagged with a HA epitope and MKK7-K76E was tagged with a FLAG epitope, expression of these epitopes should be detected by Western blot analysis if transfection was successful. However, anti-HA antibody or anti-FLAG antibody failed to detect any expression of their corresponding HA or FLAG sequence. This failure might be caused by the unsuccessful expression of these plasmids when they were amplified in E. Coli. Therefore, to demonstrate the expression of these three plasmids after amplification, the presence of these plasmids was confirmed by digestion with EcoR I. pcDNA3.1/Zeo(+) containing only one EcoR I site at 953 (Table 2) was cut into linear form and showed one band on the agarose gel at the position close to 5 kb (Fig. 16). FLAG-MKK7-K76E pCR3.1 with two EcoR I sites at 727 and 2058 (Table 2) showed two bands on the gel, one close to 5 kb and the other close to 1.3 kb (Fig. 16). HA-SEK1-AL pcDNA3.1/Zeo(+) which has three EcoR I sites at 965, 1026 and 1323 (Table 2) showed one band close to 6 kb and the other one close to 300 bp, while the third band which was expected to appear at 60 bp was too small to be separated and was not visible on the gel (Fig. 16). RT-PCR was then performed to assess the expression of these plasmids at the transcription level after transfection. The primers for both SEK1-AL and MKK7-K76E (Table 3) were selected from their corresponding plasmid sequences by using OLIGO 4.0 software. The first primer for SEK1-AL contains a DNA sequence for the HA epitope [139] and the first primer for MKK7-K76E has a DNA fragment encoding the FLAG epitope [140]. According to the design of these two pairs of primers, the positive PCR products for SEK1-AL and MKK7-K76E are 367 bp and 605 bp, respectively (Table 3). When the primers for SEK1-AL were used for RT-PCR, only the lanes
Table 2. Plasmids used in SAPK studies. The name, size and EcoR I sites of the three plasmids used in SAPK studies are listed. The expected sizes of DNA fragments after EcoR I digestion of each plasmid are also shown.

<table>
<thead>
<tr>
<th>Name of plasmid</th>
<th>Size (bp)</th>
<th>EcoR I Sites</th>
<th>Sizes after Digestion (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1/Zeo(+)</td>
<td>5015</td>
<td>953</td>
<td>5015</td>
</tr>
<tr>
<td>HA-SEK1-AL pcDNA3.1/Zeo (+)</td>
<td>6195</td>
<td>963</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1026</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1323</td>
<td>5837</td>
</tr>
<tr>
<td>FLAG-MKK7-K76E pCR3.1</td>
<td>6375</td>
<td>727</td>
<td>1331</td>
</tr>
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<td></td>
<td></td>
<td>2058</td>
<td>5044</td>
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Fig. 15. Rat mesangial cells transfected under optimal condition. Rat mesangial cells were transfected with pCMV-SPORT-βgal under optimal condition. By the method of *in situ* β-galactosidase staining [182], the transfection efficiency can be estimated by the proportion of blue (β-galactosidase positive) cells. The pictures show two areas of the same well.
Fig. 16. Digestion of the plasmids with EcoR I. After amplification, the three plasmids, pcDNA3.1/Zeo(+), HA-SEK1-AL pcDNA3.1/Zeo(+), and FLAG-MKK7-K76E PCR3.1 were digested with EcoR I for 1 h. The three plasmids, either with or without digestion, as well as λ DNA and φX174 DNA markers were then applied to a 1 % agarose gel. After running at 100 V for 30 min, the gel was photographed.
Table 3. The primers for SEK1-AL and MKK7-K76E. The upper primer for SEK1-AL contains a DNA fragment for HA epitope and the upper primer for MKK7-K76E has a DNA sequence for FLAG epitope. The expected sizes of PCR products are listed.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Expected Size</th>
</tr>
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<tbody>
<tr>
<td>SEK1-AL (+)</td>
<td>5'TACGATGTTCCAGATTACGC</td>
<td>367 bp</td>
</tr>
<tr>
<td>SEK1-AL (-)</td>
<td>5'CCATGAGAAGTGTGTTTTTG</td>
<td></td>
</tr>
<tr>
<td>MKK7-K76E (+)</td>
<td>5'CTACAAGGACGACGATGA</td>
<td>605 bp</td>
</tr>
<tr>
<td>MKK7-K76E (-)</td>
<td>5'CAAGGCAGCCACTGATGC</td>
<td></td>
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representing transfection with HA-SEK1-AL pcDNA3.1/Zeo(+) showed a strong band at the position between 300 and 400 bp, corresponding to positive product for SEK1-AL (Fig. 17, upper panel). The primers for MKK7-K76E detected a bright band at 600 bp corresponding to positive product for MKK7-K76E only in the lanes representing transfection with FLAG-MKK7-K76E pCR3.1 (Fig. 17, lower panel). These RT-PCR results confirmed the success of the transfection.

We examined the effects of kinase-inactive mutants of SEK1 and MKK7 on Cd2+-dependent SAPK activation. Kinase-inactive mutant SEK1-AL caused a small decrease in Cd2+-stimulated SAPK activity compared with the cells transfected with the empty plasmid, but this did not reach statistical significance (Fig. 18). In contrast, kinase-inactive MKK7-K76E mutant blocked 50% of Cd2+-dependent SAPK activity and reached statistical significance (p < 0.05). Therefore, MKK7 plays a more important role in Cd2+-dependent SAPK activation than SEK1 in rat mesangial cells.

3.3.3. Effects of kinase-inactive mutants of SEK1 and MKK7 on c-fos induction

We further examined the effects of kinase-inactive mutants of SEK1 and MKK7 on c-fos mRNA induced by Cd2+. However, we did not see any significant decrease in Cd2+-induced c-fos mRNA after transfecting rat mesangial cells with kinase-inactive mutants of either SEK1-AL or MKK7-K76E (Fig. 19).

Intracellular signaling is a very complicated process. Cross-talk between different pathways is not unusual. Inhibiting one pathway may enhance the effect of the other pathway. Thus, we examined the effects of kinase-inactive mutants of SEK1 and MKK7 on Cd2+-dependent ERK activation. Neither SEK1-AL nor MKK7-K76E caused any significant change in ERK activity stimulated by Cd2+ compared with the cells transfected with the empty plasmid (Fig. 20).

3.3.4. Combined effects of PD98059 and MKK7-K76E mutant on c-fos induction

Although kinase-inactive mutants of SEK1 and MKK7 did not show any inhibitory effect on Cd2+-dependent c-fos induction, we can not deny the involvement of the SAPK pathway in c-fos
Fig. 17. RT-PCR for SEK1-AL and MKK7-K76E. Mesangial cells were either transfected with vector, SEK1-AL, or MKK7-K76E, or not transfected (Control). After quiescence was induced, cells were incubated for 8 h with or without 10 μM CdCl₂. Total RNA was isolated and either 2 μg RNA or H₂O (lane 2 as control for reverse transcription) was used for reverse transcription into first strand cDNA. Two sets of PCR were then performed by using primers for either SEK1-AL (upper panel) or MKK7-K76E (lower panel). Either first strand cDNA, or H₂O (lane 1 as negative control), or plasmids (lane 11 as positive control: SEK1-AL for upper panel and MKK7-K76E for lower panel) were taken as templates. A 100 bp DNA ladder (lane 12) is also included on the gel.
Fig. 18. Effects of SEK1-AL and MKK7-K76E on SAPK activation by Cd²⁺. Mesangial cells were transfected with either vector alone, or SEK1-AL mutant, or MKK7-K76E mutant for 5 h, recovered with normal medium containing 10% FBS overnight, followed by starvation with low serum medium containing 0.4% FBS for 48 h. Cells were then incubated for 8 h in the presence or absence of 10 μM CdCl₂. Cell extracts were collected and immunoprecipitated with anti-JNK1 antibody for measurement of SAPK activity. Values are mean ± s.e. from six separate experiments. The activity in cells transfected with empty vector in the absence of CdCl₂ is taken as 100%.
Fig. 19. Effects of SEK1-AL and MKK7-K76E on c-fos induction by Cd²⁺. Mesangial cells were transfected with either vector, or SEK1-AL mutant, or MKK7-K76E mutant. After quiescence was induced by 48 h starvation, cells were incubated in the presence or absence of 10 μM CdCl₂ for 8 h. Total RNA was isolated and analyzed by Northern blotting for c-fos mRNA and corrected for loading by 18S rRNA. Values are shown as mean ± s.e. from six independent experiments and are expressed relative to the levels in cells transfected with empty vector in the absence of CdCl₂ taken as 100%.
Mesangial cells were transfected with either vector, or SEK1-AL, or MKK7-K76E. After starvation, quiescent cells were incubated with or without 10 μM CdCl₂ for 8 h. Cell extracts were collected and immunoprecipitated with anti-ERK2 antibody for measurement of ERK activity. Values are mean ± s.e. from six separate experiments and are expressed as a percentage of the activity in vector transfected cells without CdCl₂ taken as 100%.
induction by Cd\textsuperscript{2+}. To further assess the role of Cd\textsuperscript{2+}-dependent SAPK activation in c-fos induction by Cd\textsuperscript{2+}, we suppressed both the ERK and the SAPK pathways with PD98059 and MKK7-K76E mutant and observed their combined effects on Cd\textsuperscript{2+}-dependent c-fos induction. In cells transfected with the empty vector, pretreatment with 100 \( \mu \)M PD98059 caused a 15 % decrease in c-fos mRNA in response to 10 \( \mu \)M Cd\textsuperscript{2+} and reached statistical significance (Fig. 21, \( p < 0.01 \)), whereas in cells transfected with MKK7-K76E, pretreatment with 100 \( \mu \)M PD98059 significantly inhibited 30 % of c-fos mRNA in response to 10 \( \mu \)M Cd\textsuperscript{2+} (\( p < 0.005 \)). After pretreatment with 100 \( \mu \)M PD98059, 10 \( \mu \)M Cd\textsuperscript{2+} still caused a significant increase in c-fos mRNA in empty vector transfected cells (\( p < 0.0002 \)), however, it failed to do so in MKK7-K76E transfected cells. Suppression of both the SAPK and the ERK pathways via transfection with MKK7-K76E and subsequent pretreatment with PD98059 showed greater inhibitory effect on Cd\textsuperscript{2+}-dependent c-fos induction than inhibition of the ERK pathway by pretreatment with PD98059 alone (Fig. 21, \( p < 0.02 \)), confirming the involvement of the SAPK pathway in mediating c-fos induction by Cd\textsuperscript{2+}.

3.4. Effect of Cd\textsuperscript{2+} on cell viability

Since long-term exposure to 10 \( \mu \)M Cd\textsuperscript{2+} would likely be lethal to the cells, we assessed cell viability by the MTT assay after exposed to 10 \( \mu \)M Cd\textsuperscript{2+} for different periods of time. The MTT assay examines the metabolic ability of cells to convert the tetrazolium salt by mitochondrial succinate dehydrogenase into colored formazan \cite{194}, which is soluble in DMSO and can be measured spectrophotometrically at 570 nm. This process requires intact mitochondria and only occurs in living cells \cite{194}. Exposure to 10 \( \mu \)M Cd\textsuperscript{2+} within 4 h did not cause any significant change in the OD value at 570 nm (Fig. 22). However, exposure to 10 \( \mu \)M Cd\textsuperscript{2+} for 8 h or longer significantly decreased the OD value (\( p < 0.005 \)), indicating the cells lost their viability after long-term exposure to 10 \( \mu \)M Cd\textsuperscript{2+}.
Fig. 21. Combined effects of PD98059 and MKK7-K76E on c-fos induction by Cd$^{2+}$. Mesangial cells were transfected with either vector or MKK-K76E. After starvation, cells were pretreated with or without 100 μM PD98059 for 1 h, followed by incubation in the presence or absence of 10 μM CdCl$_2$ for 8 h. Total RNA was collected and analyzed by Northern blotting for c-fos mRNA. Values are shown as mean ± s.e. from six independent experiments and are expressed relative to the level in vector transfected cells stimulated by CdCl$_2$ without pretreatment of PD98059 taken as 100 %.
Fig. 22. *Cell viability after exposure to Cd\textsuperscript{2+}.* Quiescent mesangial cells were exposed to 10 µM CdCl\textsubscript{2} for the times indicated. MTT test was then performed as described in Materials and Methods. Values represent mean ± s.e. from triplicate samples. * Denotes a significant decrease under the basal level at p < 0.005.
3.5. Effects unique for Cd\textsuperscript{2+}

To determine the specificity of Cd\textsuperscript{2+} on c-fos induction in mesangial cells, we observed the effects of several different metal ions on ERK and SAPK activities, and c-fos mRNA. All the metal ions are at 10 μM and in divalent states as the chloride salts. After 8 h exposure, only Cd\textsuperscript{2+} showed large increases in all the three signals (Fig. 23), indicating Cd\textsuperscript{2+} to be a specific inducer of c-fos in mesangial cells.
Fig. 23. Specificity of responses to Cd\textsuperscript{2+}. Quiescent mesangial cells were exposed to 10 \(\mu\text{M}\) each of Cd, Co, Cu, Hg, Mg, Mn, Ni, or Zn, all in the divalent state as the chloride salt. Control cells denoted Cn were exposed to a medium change alone without any additional metals. Cells were collected at 8 h for measurement of ERK and SAPK activity, and -fos mRNA. The top two panels are autoradiograms from electrophoretic gels showing myelin basic protein phosphorylated by an anti-ERK2 immunoprecipitate and GST-c-Jun fusion protein phosphorylated by an anti-JNK1 immunoprecipitate, respectively. The bottom two panels are from a Northern blot of total RNA hybridized with cDNAs either to -fos mRNA or to 18 S rRNA which is shown to indicate uniform loading of the gel.
4. DISCUSSION

The c-fos proto-oncogene is a member of the class of immediate early genes which are rapidly induced upon stimulation of quiescent cells with growth factors or serum. Expression of the c-fos proto-oncogene has been associated with a variety of biological responses, including proliferation and differentiation [19]. Transcription of c-fos is regulated by several DNA regulatory elements: CRE [92], SRE [93], SIE [90], and HSE [94]. Each of them may be targeted by different stimuli. However, some stimuli target more than one regulatory element within the c-fos promoter, and the SRE seems to be always involved in c-fos induction by a variety of stimuli [89].

4.1. Activation of the SRE

The sequence of SRE contains the motif CC[AT]₆CG that forms the core binding site for SRF [120]. SRF binding is very important for SRE activity, because either SRE mutations preventing SRF binding or SRF depletion via antibody microinjection block the response of the SRE to growth factor stimulation [195, 196]. Adjacent to the 5' side of the SRF-binding site is an Ets domain-binding motif CAGGAT, which is recognized by TCF [120]. TCF contains an Ets domain at its N-terminal, which mediates direct DNA contact with the SRE [123, 197]. The homologous region B on TCF is required for interaction with SRF [89]. Interaction with SRE-bound SRF via the region B enables stable binding of TCF to the SRE [123, 198]. The third homologous region at the C-terminal contains several phosphorylation sites for the MAPK family [199]. Activation of the SRE requires its binding with dimeric SRF and TCF to forms the SRE ternary complex [89, 120].

4.2. Cd²⁺ and the CaMK pathway

Several members of the MAPK family activate TCF via its C-terminal which is necessary for the formation of the SRE ternary complex [109], whereas CaMK activates SRF [122, 125], another factor in this complex. CaMK can also phosphorylate CREB [200], which itself is sufficient
to induce c-fos via CRE. Cd2+ has a similar ionic radius to Ca2+ [25] and has been reported to substitute for Ca2+ in a number of Ca2+-binding proteins including calmodulin [201, 202]. Calmodulin plays an important role in modulating the intracellular concentration of Ca2+ [203]. It contains four Ca2+-binding domains. Occupancy of these domain causes activation of the Ca2+-calmodulin complex which activates a number of protein kinases including CaMK and myosin light chain kinase [204]. In vitro, Cd2+ can induce conformational changes in calmodulin and activate calmodulin-dependent targets [201-203]. However, in vivo, Cd2+ does not activate CaMK under conditions where c-fos is induced, and blocking the CaMK pathway with a CaMK inhibitor does not diminish the c-fos mRNA response to Cd2+ [18]. These observations indicate the CaMK pathway does not participate in c-fos induction by Cd2+.

4.3. Cd2+ and the ERK pathway

Since several members of the MAPK family can activate TCF [109], the MAPK signaling system appears to be the candidate to mediate c-fos induction by Cd2+ via activation of SRE. As a member of TCF, Elk1 is well established as a substrate for ERK [199]. The ERK pathway is known to be triggered by serum and TPA. The growth factors in serum enter the ERK pathway by binding with growth factor receptors, which in turn activate receptor tyrosine kinases, triggering a cascade of the downstream kinases [126]. TPA enters the ERK pathway by activating PKC, which further activates a kinase cascade of the ERK pathway [129]. Cd2+ has been reported to activate PKC in several cell lines [10, 15, 205, 206] and induction of c-fos by Cd2+ in rat kidney LLC-PK1 cells has been shown to depend on activation of PKC [17]. However, in rat mesangial cells, Cd2+ failed to activate PKC, and the PKC inhibitor calphostin C did not inhibit c-fos induction by Cd2+ [18]. Nevertheless, Cd2+ may still activate the ERK pathway in a PKC-independent manner. Although it has been reported that induction of c-fos by Cd2+ is independent of the ERK pathway in rat PC12 cells [12], we found Cd2+ significantly increased ERK activity in rat mesangial cells and ERK activity showed almost the same patterns of time course and concentration-dependent activation in response to Cd2+ as those of c-fos mRNA in response to Cd2+. We also found that
blockage of the ERK pathway with PD98059 attenuated the c-fos mRNA in response to Cd\textsuperscript{2+}. These results confirm the contribution of the ERK pathway in mediating c-fos induction by Cd\textsuperscript{2+}. However, PD98059 did not show a concentration-dependent inhibition on c-fos mRNA in response to Cd\textsuperscript{2+}. Even present at 100 \textmu M, the inhibitor only decreased c-fos mRNA level by 24%. This was not because of a failure of the inhibitor to block the ERK pathway effectively. At 50 \textmu M, PD98059 diminished the Cd\textsuperscript{2+}-stimulated ERK activity to the pre-treatment levels. These findings indicate that c-fos induction by Cd\textsuperscript{2+} is only partially mediated by the ERK pathway.

4.4. Cd\textsuperscript{2+} and the SAPK pathway

Elk1 has also been identified as an efficient substrate for SAPK [207-209]. The SAPK pathway is generally activated in response to inflammatory cytokines and environmental stresses. Since heavy metals are usually regarded as stress factors to mammalian cells, it is reasonable to take the SAPK pathway as another candidate to mediate c-fos induction by Cd\textsuperscript{2+}. Consistent with the observations of Matsuoka et al. in LLC-PK1 cells [210], we found Cd\textsuperscript{2+} increased SAPK activity in rat mesangial cells. We also observed that both time course and dose-dependent effects of Cd\textsuperscript{2+} on SAPK activity were in good agreement with those of Cd\textsuperscript{2+} on c-fos mRNA, indicating the involvement of the SAPK pathway in mediating c-fos induction by Cd\textsuperscript{2+}. SAPK is known to be activated by SEK1 through dual phosphorylation on Thr-183 and Tyr-185 [139, 142]. However, SAPK activity is still inducible in SEK1 deficient cells after osmolarity changes or UV irradiation, indicating the existence of other SAPK activators [211, 212]. Recently, a novel SAPK kinase, MKK7, was cloned and it was thought to be more specific for SAPK than SEK1 [140, 192]. We found that dominant negative mutant SEK1-AL did not cause significant decrease in Cd\textsuperscript{2+}-induced SAPK activity, whereas dominant negative mutant MKK7-K76E inhibited 50% of Cd\textsuperscript{2+}-dependent SAPK activity, indicating that SAPK activation by Cd\textsuperscript{2+} is mainly mediated by MKK7 in rat mesangial cells. However, block of the Cd\textsuperscript{2+}-dependent SAPK activation with MKK7-K76E did not attenuate Cd\textsuperscript{2+}-induced c-fos mRNA significantly. This is not due to a failure of transfection, because RT-PCR showed the success of transfection.
Western blot analysis failed to detect any expression of the HA and FLAG epitopes which were included in SEK1-AL and MKK7-K76E respectively. There are several possible reasons. First, the commercial antibodies may not have been of good quality or useful for Western blotting. Since positive controls were not available, this possibility could not be ruled out. Second, even if the antibodies were appropriate, the conformation of the expressed proteins may have prevented the epitopes being detected by these antibodies. Third, the expression levels of the proteins may have been too low to be detected by the antibodies. This seems unlikely, because the expressed MKK7-K76E showed a significant inhibition on SAPK activity induced by Cd2+.

Since cross-talk between different signaling pathways is common, inhibiting one pathway may reinforce the effect of the other pathways. Nevertheless, blockage of the SAPK pathway did not show any significant effect on Cd2+-dependent ERK activation. Therefore, the failure of MKK7-K76E to attenuate Cd2+-dependent c-fos induction may be explained by one of the following two possibilities. First, the SAPK pathway may not participate in mediating c-fos induction by Cd2+. The similar patterns of time course and dose-dependent induction of SAPK and c-fos mRNA in response to Cd2+ may be coincidental. Alternatively, the SAPK pathway may be involved in Cd2+-dependent c-fos induction. In keeping with the limitations of transient transfection, the transfection efficiency in rat mesangial cells only reached 40% when they were transfected with pCMV-SPORT-βgal under optimal conditions. Presumably the number of cells transfected with MKK7-K76E was no higher. The SAPK activity in the remaining untransfected cells could still be activated by Cd2+ and would be enough to increase c-fos transcription. However, block of both the ERK and SAPK pathways with PD98059 and MKK7-K76E showed a synergistic inhibitory effect on Cd2+-dependent c-fos induction, confirming the SAPK pathway to be another mechanism participating in c-fos induction by Cd2+.

4.5. Cd2+ and the HSE

Inhibition of both the ERK and SAPK pathways still did not prevent Cd2+-dependent c-fos induction completely. Thus, additional mechanisms may also be involved in this process. Since a
number of extracellular stimuli can induce c-fos by targeting more than one DNA regulatory element within the c-fos promoter [89], Cd²⁺ may also activate the other regulatory elements in addition to SRE. Recently, HSE has been found to exist in the human and rodent c-fos promoters and showed high responsivity to heat, arsenite, and cadmium in a number of cells [94]. Therefore, HSE seems to be another regulatory element within the c-fos promoter to regulate c-fos transcription by Cd²⁺ in addition to SRE. This possible mechanism needs to be further investigated. However, it must be noted that Ishikawa et al. [94] used a Cd²⁺ concentration of 100 μM in their study.

4.6. Biphasic activation of ERK and SAPK by Cd²⁺

The ERK and SAPK pathways are generally triggered by different extracellular stimuli. The ERK pathway is usually activated by mitogens and is responsible for cell proliferation and differentiation [213], whereas the SAPK pathway is always initiated by stressors and is thought to play an important role in apoptosis [134]. However, in some cell lines, these two pathways can be activated by the same stimuli and they may not always have opposing effects [214-216]. We found that both ERK and SAPK showed similar biphasic temporal activation in response to 10 μM Cd²⁺ with the transient increase peaking at 15 min and the prolonged phase peaking at 8 h. This finding indicates a common mechanism may be involved in these responses. Since Cd²⁺ can interfere with intracellular Ca²⁺ metabolism [7, 8] and Ca²⁺ has been reported to be required for SAPK activation by Cd²⁺ in LLC-PK1 cells [210], it seemed possible that the effects of Cd²⁺ on these two pathways could be secondary to a Cd²⁺-mediated increase in intracellular Ca²⁺ concentration. However, it was found that 8 h exposure to 10 μM Cd²⁺ only caused a small increase in cytosolic Ca²⁺ concentration from 137 ± 25 nM to 259 ± 59 nM in rat mesangial cells [181]. Therefore, it is more likely that intracellular Cd²⁺ affects these processes directly rather than being mediated by Ca²⁺. The intracellular Cd²⁺ may trigger both the ERK and SAPK pathways by activating their common upstream kinases. Ras is one of the candidates, because it has been reported to be involved in activating both the ERK and SAPK pathways [127, 135, 136]. In this thesis, we did not address this
Activation of the above two pathways involves a series of phosphorylation events. However, these events are antagonized by activation of a group of protein phosphatases [217]. The group of protein phosphatases may regulate the MAPK cascades at different levels [218]. Recently, a family of dual-specificity protein phosphatases, which can dephosphorylate both phosphothreonine and phospho-tyrosine residues of the MAPK family, has been identified and termed MAPK phosphatases (MKP) [219, 220]. Within different cell lines, MKP-1 can be induced by activation of ERK to dephosphorylate SAPK and p38 [221], or alternatively, induced by activation of SAPK or p38 to inactive ERK [222, 223]. MKP-2 inhibits ERK and SAPK preferentially [219]. MKP-3 dephosphorylate ERK specifically [224], and MKP-4 is a relatively nonspecific phosphatase for the MAPK family [225]. Therefore, the termination of the transient activation of ERK and SAPK in response to Cd^{2+} may be mediated by the family of MKP. When rat mesangial cells are exposed to 10 μM Cd^{2+}, Cd^{2+} binds to the cell surface followed by rate-limiting internalization with a V_{max} of 3.8 μmol/g protein/h [176]. The intracellular ionic Cd^{2+} concentration increases until it is sequestered by induction of metallothionein after exposure to 10 μM Cd^{2+} for 4 h [178], which keeps the cytosolic concentration of Cd^{2+} at about 1 pM [181]. Although induction of metallothionein prevents further increase in cytosolic Cd^{2+} concentration, intracellular cadmium continues to accumulate [176], which may inhibit the MKP family through some unknown mechanisms and cause sustained activation of ERK and SAPK. A number of authors have reported that inhibition of protein phosphatases causes sustained activation of ERK and SAPK [217, 218].

### 4.7. Biphasic induction of c-fos by Cd^{2+}

As one of the immediate early genes, c-fos expression is usually rapid and transient, and is essential for cell proliferation [19]. However, we found 10 μM Cd^{2+} caused biphasic induction of c-fos in rat mesangial cells with an immediate early peak at 30 min and a prolonged late peak at 8 h. In contrast, 1 μM Cd^{2+} only induced a single transient peak of c-fos induction which is similar as the first peak induced by 10 μM Cd^{2+}. The pattern of the transient c-fos induction is also observed
in rat mesangial cells after stimulation with serum or TPA [190]. Since mitogen-induced c-fos expression is well known to be related to cell proliferation, we may infer that Cd^{2+}-stimulated transient c-fos induction is also related to cell proliferation. Actually, Cd^{2+} has been confirmed to stimulate cell proliferation in a number of different cells [226, 227]. However, sustained c-fos expression is related with apoptosis [24]. The MTT assay in this thesis showed that cell viability began to decrease after exposure to 10 μM Cd^{2+} for 8 h. This finding is consistent with the observation that, in LLC-PK1 cells, 8 h exposure to 10 μM Cd^{2+} decreased cell viability assayed by trypan blue exclusion [228] and caused DNA fragmentation [229]. Thus, sustained c-fos induction by 10 μM Cd^{2+} may be associated with apoptosis. The dose-dependent effect of Cd^{2+} on c-fos induction showed that lower than 3 μM Cd^{2+} caused similar transient increase in c-fos mRNA whereas higher than 3 μM was required to stimulate prolonged c-fos induction. Therefore, we propose that Cd^{2+} stimulates proliferation of mesangial cells at low micromolar doses, whereas prolonged exposure to elevated concentrations of Cd^{2+} induces apoptosis. In healthy human kidney, the mesangial cells are usually quiescent and the equilibrium between proliferation and apoptosis is well balanced [164]. Proliferation of the mesangial cells followed by pathological stimuli represents one of the major features of glomerular injury and often heralds sclerosis and loss of glomerular function [167-169]. Chronic occupational exposure to cadmium causes renal dysfunction in some of the exposed individuals and Cd^{2+} concentrations in the micromolar range occur in their plasma filtrate [230]. The initial increase of c-fos by low micromolar doses of Cd^{2+} might be responsible for proliferation of mesangial cells and subsequent glomerular sclerosis, whereas the sustained increase in c-fos by elevated concentrations of Cd^{2+} might be associated with progressive loss of mesangial cells, which in turn leads to mesangial replacement and loss of functioning nephrons. The specific effects of Cd^{2+} on ERK and SAPK activation as well as c-fos induction among a number of divalent metals indicate a unique role of Cd^{2+} in mesangial cell toxicity.
4.8. Summary and significance

The conclusions that can be drawn from this study are: (i) Cd\(^{2+}\) is a specific inducer of c-fos in mesangial cells. (ii) Induction of c-fos by Cd\(^{2+}\) is mediated by both the ERK and SAPK pathways. (iii) Less than 1 μM Cd\(^{2+}\) causes a transient increase in c-fos mRNA as well as ERK and SAPK activity, whereas over 3 μM Cd\(^{2+}\) stimulates a sustained increase in c-fos mRNA and ERK and SAPK activity in addition to the initial phase.

Since cadmium is an important environmental pollutant, a number of investigators have focused on the molecular mechanism of cadmium toxicity and carcinogenicity, especially the induction of proto-oncogenes by cadmium. As one of the proto-oncogenes, c-fos expression has been associated with a number of important cellular events, including proliferation, differentiation, and apoptosis [19, 23]. Cadmium has been reported to induce c-fos in a number of cells [13, 15-18], but the mechanism of c-fos induction by cadmium is poorly understood. In this thesis, we address the mechanism of c-fos induction by Cd\(^{2+}\) in mesangial cells, identifying two family members of the MAPK cascades, the ERK and SAPK pathways, as mediators responsible for Cd\(^{2+}\)-dependent c-fos induction.

Stimulation of cell proliferation is one of the important carcinogenic properties of cadmium. However, the effect of cadmium on cell proliferation remains controversial. Cadmium stimulates growth in some cell types but is inhibitory in others [12]. Our results suggest that the effect of Cd\(^{2+}\) on cell proliferation may be related to the dose and time of exposure, transient exposure to low micromolar concentrations of Cd\(^{2+}\) stimulating cell proliferation whereas prolonged exposure to more elevated doses inducing cell death.

Cadmium can induce either cell proliferation [12] or apoptosis [17, 20-22] in different cell lines. Our data raise the possibility that these different effects of cadmium may be associated with the pattern of c-fos expression and mediated by the ERK and SAPK pathways. This suggests avenues for further exploring regulatory mechanisms of mitogenesis and apoptosis.
4.9. Future directions

A number of interesting questions have been raised from this thesis that are worth further investigation.

We have confirmed that both the ERK and SAPK pathways are involved in Cd\textsuperscript{2+}-dependent c\textsuperscript{-}fos induction. However, inhibition of both the ERK and SAPK pathways with PD98059 and MKK7-K76E does not block c\textsuperscript{-}fos induction by Cd\textsuperscript{2+} completely. As the third member of the MAPK cascades, the p38 pathway is known to be triggered by a number of stressors [134]. Furthermore, p38 can phosphorylate and activate Elk1 in some cell lines [138]. The p38 pathway may also be involved in mediating Cd\textsuperscript{2+}-dependent c\textsuperscript{-}fos induction. Testing this hypothesis may provide the full picture of the relationship between the MAPK cascades and c\textsuperscript{-}fos induction by Cd\textsuperscript{2+}.

Transcription of c\textsuperscript{-}fos can be mediated by several regulatory elements within the c\textsuperscript{-}fos promoter [89]. SRE is a pivotal regulatory element that is always involved in c\textsuperscript{-}fos induction by a large variety of stimuli [89]. However, it has been reported recently that the human and rodent c\textsuperscript{-}fos promoters contain HSE which is highly responsive to cadmium in a number of cells [94]. Therefore, the involvement of HSE in mediating Cd\textsuperscript{2+}-dependent c\textsuperscript{-}fos induction needs to be further investigated.

The present study identifies that both the ERK and SAPK pathways are activated by Cd\textsuperscript{2+} and are each partially responsible for Cd\textsuperscript{2+}-dependent c\textsuperscript{-}fos induction. Cd\textsuperscript{2+} does not seem to affect ERK and SAPK activation directly, because inhibition of their upstream kinases blocks their activation by Cd\textsuperscript{2+}. Further study of the effects of Cd\textsuperscript{2+} on the upstream kinases of the MAPK family at the MAPKK level (MEK1, MEK2, SEK1, and MKK7), MAPKKK level (Raf1 and MEKK1), and G protein level (Ras, Rac, and Cdc42) will elucidate how Cd\textsuperscript{2+} enters the MAPK cascades and subsequently induces c\textsuperscript{-}fos transcription.

It has been shown in this study that 10 μM Cd\textsuperscript{2+} causes biphasic activation of both ERK and
SAPK with a transient peak at about 15 min and a prolonged peak about 8 h later. Activation of the MAPK family is downregulated by a group of dual specificity phosphatases, termed the MKP family [217, 218]. The termination of the transient activation of ERK and SAPK in response to Cd$^{2+}$ could be mediated by activation of the MKP family. Since a number of studies have shown that inhibition of the MKP family causes prolonged activation of ERK and SAPK, we may further hypothesize that prolonged exposure to 10 μM Cd$^{2+}$ inhibits the MKP family and subsequently causes sustained activation of ERK and SAPK. Investigating the effect of Cd$^{2+}$ on the MKP family might provide more important information regarding the molecular mechanism of cadmium toxicity as well as regulatory mechanism of both the ERK and SAPK pathways.

We also found that 10 μM Cd$^{2+}$ caused a sustained induction of c-fos and activation of ERK and SAPK, whereas exposure at this level for more than 8 h significantly decreases cell viability. Probably the cells undergo apoptosis after exposure to 10 μM Cd$^{2+}$ for more than 8 h and the sustained activation of the above three signals may be associated with this process. To confirm that the cells progress to apoptosis, we would need to observe specific markers for apoptosis such as DNA fragmentation [83]. The relationship between the sustained activation of the three signals and apoptosis might be studied by observing specific markers for apoptosis. This might be done by blocking the ERK and SAPK pathways separately and testing each of their specific effects on apoptosis by observing the specific markers. Such results might elucidate the mechanism by which Cd$^{2+}$ induces apoptosis, the role of the ERK and SAPK pathways in regulating apoptosis, and the relationship between sustained induction of c-fos and apoptosis.
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