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Free Radicals in the Ischemia/Reperfusion Injury of Liver

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Abbreviations

eNOS:	Endothelial nitric oxide synthase
GPx:	Glutathione peroxidase
HIF-1:	Hypoxia inducible factor-1
H_2O_2:	Hydrogen peroxide
HPH:	HIF-1 prolyl hydroxylase
IL-1:	Interleukin-1
iNOS	Inducible nitric oxide synthase
I/R:	Ischemia/reperfusion
nNOS	Neural nitric oxide synthase
$NO^{\bullet}$:	Nitric oxide
$O_2^{\bullet-}$:	Superoxide
$OH^{\bullet}$:	Hydroxyl radical
$ONOO^-$:	Peroxynitrite
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
Stat3:	Signal transducer and activator of transcription-3
TF:	Transcription factor
TNF-α:	Tumor necrosis factor α
XDH:	Xanthine dehydrogenase
XO:	Xanthine oxidase

Outline

Abstract

Ischemia/reperfusion (I/R) injury is one of the primary causes of poor graft function in liver transplantation. The destructive effects of I/R are mainly due to the generation of reactive oxygen species (ROS) following the reoxygenation process. These reactive molecules can cause direct tissue damage and initiate a series of detrimental cellular signaling events, which lead to inflammation, apoptosis, and eventually, organ failure. This review tends to summarize the current understanding of ROS generation and their harmful effects during the liver I/R injury. The cellular responses to I/R injury and the potential therapeutic approaches are also addressed.

Introduction

The ischemia/reperfusion (I/R) condition often occurs in diseases such as stroke and cardiac infarct, or during the clinical interventions such as coronary bypass and organ transplantation [1, 2]. During the I/R process, a transient ischemia followed by a reperfusion of blood or oxygen usually causes severe injuries to the organ. The injuries include inflammation, cell death, or even organ failure. A better understanding of the mechanism of I/R injury will help to design therapeutic strategies for ameliorating the tissue damage cause by I/R and will aid in increasing the success rate of organ grafts. Recently, accumulating evidences have suggested that many pathophysiological events during the I/R injury are mediated by the generation of some reactive radical or non-radical molecules [3, 4]. Such molecules include the reactive nitrogen species (RNS) and the reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). These reactive molecules can lead to tissue damage through direct oxidation of biomolecules such as DNA, lipid, and protein. Some molecules can also act as second messengers in the signaling cascades controlling cell fates and/or triggering inflammatory reactions [3, 4]. This review will focus on the generation and roles of these reactive molecules during I/R injury of the liver, as well as the molecular and cellular events in the pathophysiological process.

I/R Injury of Liver

Liver transplantation is broadly used as a therapeutic approach in the treatment of both acquired and inherited liver disorders. Currently, the acute graft failure and long-term allograft rejection are still two major concerns in the clinical application of liver transplantation [5]. It has been well accepted that the acute I/R injury after the operation is responsible for the initial poor

function of the grafted liver. Additionally, though the exact mechanism is not clear, the I/R tissue damage is also considered as a risk factor for the long-term survival of the grafted organ [6].

Generally, the liver I/R injury occurs in a biphasic pattern, which includes an acute- and a subacute-phase response [4]. The acute-phase, which occurs around 3-6 h after reperfusion, is remarked by the injury of hepatocytes. A burst of ROS generation is observed during this acute phase, and antioxidant enzymes and chemicals have demonstrated partial protection against the acute injury [7]. The sources of cellular ROS generation are suggested to include the xanthine/xanthine oxidase (XO) system and the mitochondria [3, 4], which will be discussed in detail in later sections. In addition, the activations of Kupffer and T-cells also happen in this phase. They are believed to play important roles in initiating the later inflammatory responses [4, 8].

About 18-24 h post reperfusion, the I/R injury enters its subacute-phase, which is characterized by massive infiltration of neutrophils [9]. These neutrophils are recruited from the circulation through a complex series of interactions with cytokines and chemoattractants released from the liver [10]. Inside the liver, Kuffer cells and the activated neutrophils are known to release a large amount of ROS molecules, which further aggravate the oxidative stress. The results of these processes are irreversible tissue damage characterized by sinusoidal obstruction as well as apoptosis and necrosis of hepatocytes.

A lot of cellular events occur during these two phases of liver I/R injury, including hepatocytic apoptosis, chemoattractants production from endothelia or hepatocytes, cytokines production by Kuffer and T cells, induction of adhesion molecules on the surfaces of endothelia and leucocytes, activation of macrophage and neutrophil by CD4 T cells, and so on [4, 11]. All these events are realized and regulated through a number of complicated cellular signaling

cascades. Recent studies have found that free radicals, especially ROS, participate in these signaling cascades by acting as the primary causes and/or secondary messengers [4, 11].

Reactive oxygen species in liver I/R injury

Traditionally, reactive oxygen species are considered as by-products or intermediates of normal metabolic reactions and processes. Although recent evidence has revealed that cells can "purposefully" produce ROS molecules (*e.g.* superoxide by NADPH oxidase) for "good use" [12], accumulation of such molecules will cause inevitable damage to the cell, mainly by oxidizing important biomolecules. During evolution, the cells have developed a number of defending mechanisms against these reactive molecules **(Figure 1)**. These mechanisms involve antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and so on. However, during environmental injuries such as liver I/R, the level of ROS generation exceeds the handling capacity of the cellular antioxidant system, leading to severe pathophysiological outcomes. The sources for this ROS burst during liver I/R injury include the xanthine oxidase system, inflammatory leucocytes, mitochondria, *etc*.

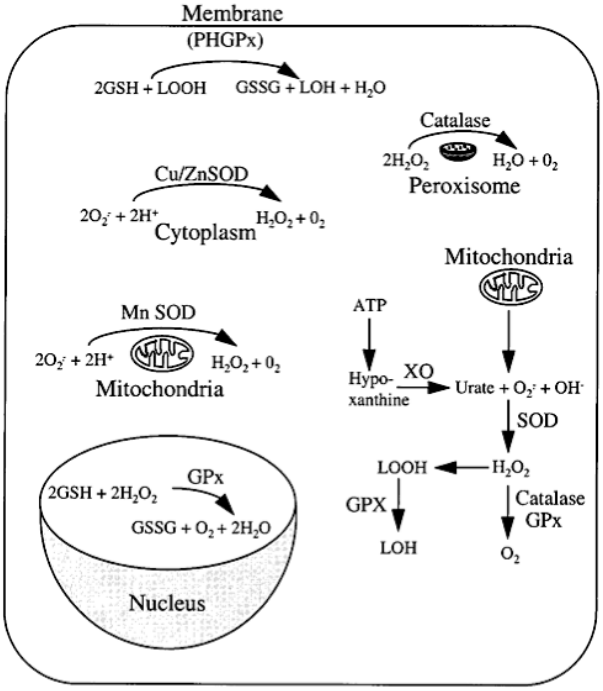

Figure 1. The intracellular ROS generation and clearance systems during I/R. The cytosolic XO system and the mitochondria are two major sources of intracellular ROS during I/R injury. The antioxidant systems include SOD, catalase, GPx, *etc.* These enzymes have various forms at different locations. SODs converts superoxide to hydrogen peroxide while catalase and GPx detoxify peroxide compounds. Adapted from [4].

The xanthine oxidase system

The mammals have two forms of xanthine oxidoreductase, namely xanthine dehydrogenase (XDH) and xanthine oxidase (XO). Though both enzymes convert hypoxanthine to xanthine and then to uric acid, the products of their reactions are different. XDH transfers two electrons from hypoxanthine/xanthine to NAD^+ and makes NADH, while XO prefers oxygen as acceptor to generate superoxide [13]. XDH can be converted into XO by proteolytic cleavage or oxidation of its specific cysteine residues. This XO system is highly abundant in the endothelial cells in liver and intestine and is related to the ROS generation during the liver I/R injury. During the ischemia stage, hypoxanthine accumulates in the liver due to the depletion of ATP. In parallel, XDH is converted into XO by certain proteolytic enzymes, which are activated during the hypoxia stage. Upon reperfusion, the accumulated hypoxanthine provides a massive amount of electrons for the XO to make superoxide from the resumed oxygen supply **(Figure 2)**. This burst of superoxide and its ROS derivates are believed to have important roles in the acute injury of liver.

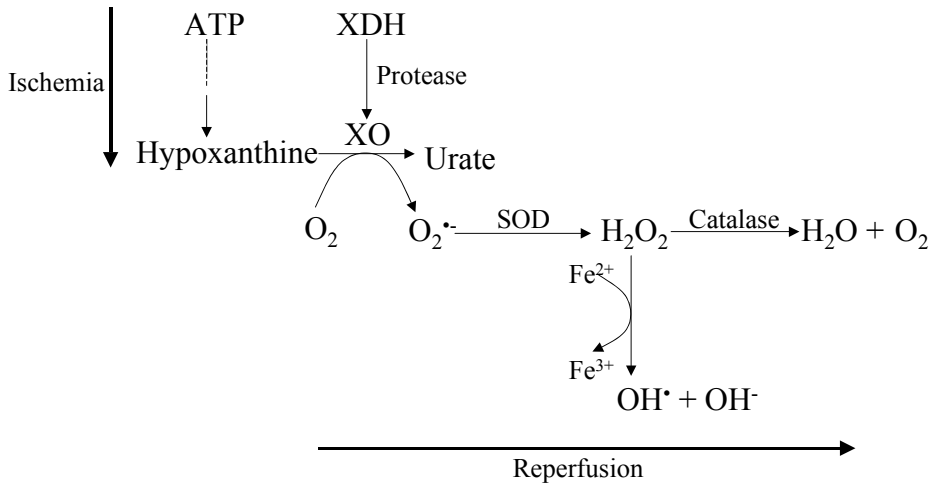

Figure 2. ROS generation by the xanthine/XO system during liver I/R injury. During the ischemia phase, ATP is converted into hypoxanthine and XDH is converted into XO. Upon reperfusion, XO will use oxygen as an electron acceptor to generate superoxide, which can then be transformed into hydrogen peroxide by SOD. In the presence of iron, hydroxyl radical is generated from hydrogen peroxide.

Interestingly, research has shown that the XO can be secreted from liver endothelial cells and travel to other sites in the body through the circulation. This may account for ROS generation and damage of other organs distal to the liver, such as the lung [14]. The importance of such circulating XO has been highlighted by the findings that circulating ROS scavenger such as SOD and catalase can decrease the injury of other organs following reperfusion [15].

Recently a procedure called "preconditioning" has been found to protect the graft from the I/R injury after transplantation [11]. During preconditioning, the organ experiences an I/R cycle when it is still in the donor body, which is called the warm ischemia. This procedure reduces the ROS generation and I/R injury of liver following the ischemia during the surgery (the "cold ischemia"). It has been shown that both xanthine accumulation and XDH to XO conversion are significantly reduced after preconditioning [16]. Furthermore, administration of xanthine and XO into the preconditioned rats abolishes the protective effects [16]. These findings support the importance of XO system in the ROS generation during the liver I/R injury. However, it is still unclear about how the cells are "trained" to limit the xanthine and XO level after preconditioning. The elucidation of these mechanisms will help to design intervening strategies to reduce ROS production from the XO system.

Leucocytes and NADPH oxidase

The major leucocytes that produce ROS duing liver I/R injury are Kupffer cells and neutrophils [4, 11]. Kupffer cell is a subset of macrophage residing in the liver, while neutrophils are recruited from circulation during the I/R injury. Both cell types are able to produce a massive amount of ROS molecules and are acting as the inflammatory source of ROS during the subacute-phase of liver I/R injury [4, 11]. Activated complement factors during reperfusion have been identified as the key mediators of the activation of Kupffer cells [17]. Inhibition of

complement factors can decrease Kupffer cells activation and attenuate the oxidant stress and reperfusion injury of liver. The recruitment and activation of neutrophils are reported to depend on the actions of a series of cytokines such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1) and chemoattractants. The sources of these factors are mainly Kupffer cells and hepatocytes [18, 19].

The major source of ROS generation in Kupffer cells and neutrophils is NADPH oxidase, an enzyme complex that transfers electrons from NADPH to oxygen and make superoxide. The key functional component of this complex is gp91phox (also known as Nox2), the activity of which is regulated by a small GTP bind protein Rac1. It has been shown that NADPH oxidase deficiency ($gp91phox^{-/-}$), as well as dominant negative form of Rac1 (N17Rac1), can reduce the post-reperfusion ROS generation and the I/R injury of liver [20, 21]. Recently, it was reported that endothelial cells and hepatocytes also possess certain isotypes of NADPH oxidase [22, 23]. Whether these non-inflammatory NADPH oxidases contribute to the ROS generation during liver I/R injury is still unclear. ROS from these sources may be involved in the signal transduction in the responses of endothelial cells and hepatocytes toward the I/R injury.

Mitochodria as a source of ROS during liver I/R injury

Serving as the major cellular compartment that consumes oxygen and generates energy, mitochondria are believed to be the primary site of ROS generation under normal physical conditions. In addition, the damage of mitochondria is also known as an important source for apoptosis. Therefore, it is postulated that mitochondria may play important roles in tissue damage during I/R injury [24]. However, there has been no agreement about the exact roles of mitochondria in the ROS generation during the I/R injury of liver [25]. One model argues that mitochondria mainly generate ROS at late stages of I/R injury after a long period of ischemia,

whereas the XO system is responsible for the short-term oxidant stress [25]. This theory is supported by the observations that more than two hours of ischemia was required for the dysfunction of mitochondria, which then led to oxidant stress [26]. However, these studies were performed on "warm ischemia" models and they underestimated the oxidant stress in mitochondria prior to severe dysfunction. A recent study using fluorescence probes has detected a sublethal oxidant stress in liver mitochondria after shorter ischemia periods [27]. Additionally, MnSOD, the superoxide scavenger that only exists in the matrix of mitochondria, was increased at both protein and mRNA levels in hepatocytes following I/R [28]. This upregulation was realized through the action of the transcription factor (TF) signal transducer and activator of transcription-3 (Stat3). These data suggested that the roles of mitochondrial ROS during liver I/R injury are still not fully appreciated. In the last section of this review, hypotheses will be proposed to study this topic.

Mechanisms of ROS-induced injury during liver I/R

Due to their highly reactive property, ROS are able to react with biomolecules such as protein, lipid, and DNA. This forms the fundament of their toxic activities. It has been postulated that during liver I/R, ROS can cause damage through lipid peroxidation, mitochondria permeabilization, and so on [25].

One popular theory about the ROS toxicity is damage of cell membrane by lipid peroxidation, which can lead to the disruption of ion homeostasis, cell swelling, and cell necrosis. Many antioxidant treatments have demonstrated ability to reduce the lipid peroxidation and cell damage by I/R [25]. However, there are still disagreements about whether the lipid peroxidation is a cause or only a consequence of cell death [29]. It has been reported that the level of hepatic lipid peroxidation following liver I/R is only about 1/10 of the level that can

cause cell death [30]. Therefore in the quantitative means lipid peroxidation by ROS may not directly cause cell death, but its products may contribute to further amplification of the inflammatory response.

The roles of mitochondria damage in triggering apoptosis have been well known. It is known that extracellular ROS from leucocytes can cause an increase of cytosolic calcium, which then enters mitochondria and leads to its dysfunction [31, 32]. The net result of this process is formation of permeability transition pores, loss of the inner mitochondrial membrane potential, and mitochondrial swelling. The leakage of mitochondrial cytochrome into the cytoplasm will initiate apoptosis cascades. In addition, more ROS molecules could be potentially released into the cytoplasm, aggravating the oxidative stress [25]. This model highlights the importance of mitochondria in the subacute-phase damage since it requires the activity of leucocytes. It has been shown that prevention of mitochondrial permeability transition could protect against liver I/R injury [25]. However, this model does not discuss the potential roles of mitochondria in the acute-phase I/R response without significant leucocytes infiltration.

ROS and the signal transduction during liver I/R injury

As we discussed before, liver is exposed to ROS from both extra- and intracellular sources during I/R injury. ROS from both sources are known to have roles in affecting cellular signal transduction pathways. The cellular redox state controls the “on and off” switch of many transcription factors, including NF-κB, AP-1, HIF, *etc* [4, 11]. These transcription factors affect cell growth and/or apoptosis by inducing the expression of genes of different categories. Understanding these processes will help us to develop intervening strategies for liver I/R injury.

NF-κB

The NF-κB transcriptional factor family has five distinct members, namely p50, p52, p65 (RelA), c-Rel, and RelB. Homo- or heterodimerization between the members forms the functional NF-κB complex to activate transcription [33]. The predominant dimer form is between p50 and p65. Normally in the cytoplasm, NF-κB is bound to proteins of the IκB family, which includes IκBα, IκBβ, and IκBγ. This association prevents NF-κB from entering the nucleus and activating transcription. Dissociation of NF-κB from IκB requires the activation of IκB-kinase (IKK), which phosphorylates IκB and leads to its degradation. IKK can be activated by many factors, including I/R, TNF-α, IL-1, *etc*. The role of ROS in NF-κB regulation is reflected by the observation that overexpression of MnSOD reduced NF-κB activation following I/R and decreased apoptosis of hepatocytes [34]. These data also suggest the pro-apoptotic effect of NF-κB during liver I/R. However, another study reported a dual phase activation of NF-κB and inhibition of the second wave resulted in increased liver damage [35]. This study indicated a dual role of NF-κB in liver I/R injury: both pro- and anti-apoptotic. The discrepancy could be due to different animal model and I/R treatment.

AP-1

The AP-1 family consists of three subgroups: the Jun proteins (v-Jun, c-Jun, JunB JunD), the Fos proteins (v-Fos, c-Fos FosB, Fra1, Fra2), and the activating transcription factors (ATF2, ATF3, B-ATF). Specific homo- or heterodimers between members of these subgroups are the functional form of AP-1 [36]. AP-1 regulates the expression of genes that are related to survival and apoptosis. It has been reported that the DNA binding domain of Jun has a conserved cysteine residue, and the oxidative status of this cysteine affects its activity [37]. Additionally, the phosphorylation status of Jun, which is critical for its activation, can also be changed by

oxidants. The enzyme that phosphorylates c-Jun, namely the c-Jun N-terminal kinase, is potentially the primary target of the increased ROS during I/R [4]. Therefore AP-1 could be a pathway by which ROS affect the fate of hepatocytes during liver I/R.

Hypoxia inducible factor-1 (HIF-1)

The target genes of HIF-1 are mainly related to angiogenesis and glycolysis [38]. Normally HIF-1 is constitutively expressed in the cell and hydroxylated by an enzyme called HIF-1 prolyl hydroxylase (HPH). This hydroxylation leads to its polyubiquitination and degradation, so normally there is a very low cellular level of HIF-1 [39]. The activity of HPH depends on O_2, iron, ascorbate, and α-ketoglutarate, so the hypoxia resulted from ischemia will decrease HPH activity and increase the level of HIF-1.

Nitric oxide in liver I/R injury

Another radical molecule involved in liver I/R injury is nitric oxide ($NO^{\bullet}$). The roles of $NO^{\bullet}$ are generally considered to be protective, since it can dilate blood vessels and alleviate inflammatory infiltration [4]. $NO^{\bullet}$ can also act as a scavenger of ROS. For instance, reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$ leads to the formation of peroxynitrite ($ONOO^-$). Although the role of $ONOO^-$ is complicated, overall $NO^{\bullet}$ can help reduce the damage of oxidant stress [40]. There are three enzymatic systems in mammals that produce $NO^{\bullet}$ from L-arginine, namely neural nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). In the liver, eNOS and iNOS are the two major isoforms of NOS [4]. Research using eNOS and iNOS deficient mice has found that these two enzymes are both protective for the post-ischemic liver, but the pattern and mechanism of their actions are distinct [41]. Both knockout strains had more severe liver injury when compared to wild type mice. At 1 h post-I/R,

inflammatory cytokines such as TNF-α was greatly increased in eNOS, but not iNOS deficient mice when compared to the wild type control. These data suggest that $NO^{\bullet}$ from these two enzymes may have different targets.

Hypothesis and experimental design

From the above review, we can conclude that free radicals, especially ROS, play important roles in the I/R injury of liver. During the two phases of injury, ROS are generated from different sources, including XO system, leucocytes, mitochondria, and so on. The ROS from mitochondria have been suggested to affect many important processes during the subacute-phase of liver I/R injury, such as signal transduction, apoptosis, and necrosis. However, very little is known about how the mitochondrial oxidative balance is changed during the acute-phase (early stage) of I/R injury and how it might affect the cell fate. My model of mitochondrial effects in the acute-phase includes the following pathway:

1) As soon as the liver is reperfused, the oxidative stress in mitochondria increases.
2) A certain amount of mitochondrial ROS is released into the cytoplasm.
3) These mitochondrial ROS are involved in the activation of different transcription factors.
4) Transcription factors increase the production of pro-inflammatory cytokines such as TNF-α from the hepatocytes.
5) Transcription factors may also increase the expression of anti-oxidant enzymes, such as CuZnSOD, MnSOD, catalase, *etc*.

In the rest of this article, I will design experiments to test this model.

Hypothesis 1: Overexpression of MnSOD will delay the inflammatory response following liver I/R.

Experimental design: Adenovirus encoding for mouse MnSOD will be used to infect mice livers through the portal vein. Control mice will be infected with adenovirus encoding for LacZ. After

the expression of transgene, I/R operation will be performed on the liver. Following I/R, mice will be sacrificed at different time points and the levels of serum TNF-α, IL-1 and chemoatrractants will be monitored. The inflammatory infiltration and apoptosis in the liver will also be examined.

Anticipated results: I expect to see decreased and/or delayed levels of cytokines and chemoattractants in the mice with MnSOD overexpression in the liver. The inflammatory infiltration and apoptosis will also be accordingly reduced in these mice. Since MnSOD is only expressed in the mitochondria and helps to decrease mitochondrial oxidative stress, such results will support my hypothesis and model.

Potential problem: The xanthine/XO system produces a large amount of ROS during the acute-phase. Though a major part of ROS in this system is generated in the circulation, it may add significant noise to the experiment and make the data hard to interpret. If this were the case, an inhibition of the XO system (*e.g.* by siRNA) might be necessary.

Hypothesis 2: Decrease of MnSOD will aggravate the inflammatory response following liver I/R.

Experimental design: Adenovirus encoding for a siRNA against mouse MnSOD will be used to infect mice livers through the portal vein to knock down the expression of MnSOD. Control mice will be infected with adenovirus encoding for a non-functional RNA. The inhibition of MnSOD will be confirmed by Northern and Western blot. I/R operation will be performed on the liver at the maximal level of inhibition. Following I/R, mice will be sacrificed at different time point and the levels of serum TNF-α, IL-1 and chemoatrractants will be monitored. The inflammatory infiltration and apoptosis in the liver will also be examined.

Anticipated results: I expect to see increased and/or accelerated levels of cytokines and chemoattractants in the mice with inhibited MnSOD in the liver. The inflammatory infiltration and apoptosis will also be accordingly aggravated in these mice. Since low level of MnSOD will primarily increase the oxidative stress in the mitochondria, such results will support my hypothesis and model.

Potential problem: the siRNA may not work very efficiently, so a screening for the suitable construct is necessary.

Hypothesis 3: NFκB activity is regulated by mitochondrial ROS in the acute-phase.

Experimental design: A luciferase transgene driven by a NFκB-responsive promoter will be delivered to the liver by adenovirus. Overexpression and knockdown of MnSOD will be performed. Control mice will be set up as being stated in the above. I/R will be performed and the liver of mice will be collected at different time points. The liver NFκB activity will be reflected by the luciferase activity.

Anticipated results: I expect to see increased and/or accelerated NFκB activity following I/R in the MnSOD knockdown mice. Meanwhile, I expect to see decreased and/or delayed NFκB activity following I/R in the MnSOD overexpression mice. NFκB regulates the expression of many pro-inflammatory genes, and such results will support my hypothesis and model.

Potential problem: NFκB may not be the right transcription factor that is regulated by mitochondrial ROS. If that were the case, its activity would not be affected by MnSOD level. If this happened, I would examine the activity of other transcription factors, such as the AP-1.

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