Metallothionein Inhibits Peroxynitrite-induced DNA and Lipoprotein Damage*

Received for publication, August 30, 2000, and in revised form, October 18, 2000. Published, JBC Papers in Press, October 20, 2000, DOI 10.1074/jbc.C000593200

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Previous studies have demonstrated that metallothionein functions as an antioxidant that protects against oxidative DNA, protein, and lipid damage induced by superoxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide. The present study was undertaken to test the hypothesis that metallothionein also protects from DNA and lipoprotein damage induced by peroxynitrite, an important reactive nitrogen species that causes a diversity of pathological processes. A cell-free system was used. DNA damage was detected by the mobility of plasmid DNA in electrophoresis. Oxidation of low density lipoprotein was measured by a thiobarbituric acid-reactive substance, which was confirmed by lipid hydroperoxide assay. Plasmid DNA damage and low density lipoprotein oxidation were induced by 3-morpholinosydnonimine, which produces peroxynitrite through the reaction between nitric oxide and superoxide anion or by synthesized peroxynitrite directly. DNA damage by 3-morpholinosydnonimine was prevented by both metallothionein and superoxide dismutase, whereas the damage caused by peroxynitrite was prevented by metallothionein only. The oxidation of low density lipoprotein by 3-morpholinosydnonimine and peroxynitrite was also significantly inhibited by metallothionein. This study thus demonstrates that metallothionein may react directly with peroxynitrite to prevent DNA and lipoprotein damage induced by this pathological reactive nitrogen species.

Oxidative damage to different cellular components makes a major contribution to many pathogeneses (1, 2). Metallothionein (MT) is a ubiquitous, low molecular weight, and highly inducible protein (3–5) that has been shown to function as an antioxidant (3, 4). Several studies have demonstrated that MT is able to quench a wide range of reactive oxygen or nitrogen species (ROS or RNS) including superoxide (O2•−), hydrogen peroxide (H2O2), hydroxyl radical (HO•), and nitric oxide (NO) at a higher efficiency than other well known antioxidants such as GSH, SOD, and catalase (6–8). An important RNS is peroxynitrite (ONO•O−), which has been shown to play a key role in many pathogeneses. Whether MT reacts with ONOO− is unknown.

Many studies have demonstrated that exposure of cells to 3-morpholinosydnonimine (SIN-1) or directly to ONOO− caused apoptotic cell death through an activation of p38 MAPK (9–11). There is also increasing evidence indicating that ONOO− causes mitochondrial structural changes, leading to the release of cytochrome c and thereby activating caspase-3 and causing apoptosis (12–14). It is well known that the cardiac toxicity of doxorubicin (DOX), an important anticancer agent, is mainly due to the formation of ROS and RNS (4). Among these reactive free radicals derived from DOX, ONOO− has been considered as the major species that leads to the oxidative damage (15, 16). Because MT inhibits both DOX-activated p38 MAPK and DOX-induced apoptosis in cardiomyocytes (17), it is possible that MT reacts with ONOO−.

The present study was undertaken to test the hypothesis that MT directly protects from ONOO−-induced oxidative damage. A cell-free system was used to avoid the influence of other cellular enzymes and antioxidants and the interaction of ONOO− with other ROS and RNS in the cell. DNA damage and oxidation of LDL were determined as indexes of ONOO−-induced oxidative damage, because DNA is a sensitive target (18, 19), and lipoproteins are also sensitive to ONOO−-induced oxidative damage (20, 21).

MATERIALS AND METHODS

Chemicals and Phosphate Buffer Preparation—MT, SOD, LDL, pTz 18U plasmid DNA, agarose, and SIN-1 and all other chemicals were purchased from Sigma. Phosphate buffer was prepared to contain 50 mM sodium phosphate, 10 mM NaCl, 0.1 mM diethylthiocarbamatesuccinic acid, pH 7.4 (21). To eliminate the contamination of transition metals such as copper and iron, the phosphate buffer was treated by Chelex 100 according to the instruction manual from Bio-Rad. Lipid hydroperoxide assay kit was obtained from Cayman Chemical (Ann Arbor, MI).

Synthesis of Peroxynitrite—Peroxynitrite was synthesized from nitrite and H2O2 in an acidic medium and rapidly quenched in NaOH as described previously (22). The solution was frozen at −20 °C, and ONOO− concentrated in the upper layer was collected. Its concentration was measured at 302 nm and calculated with a molar extinction coefficient of 1670 s cm−1 (22).

Peroxynitrite Treatment and Analysis of DNA Strand Breaks—DNA strand breaks in supercoiled DNA were analyzed after agarose gel electrophoresis as described previously (19). pTz 18U plasmid DNA (0.5 μg) was treated with 0.1–1.0 mM SIN-1 at 37 °C or with 0.05–1.0 mM ONOO− at room temperature (22–23 °C) in phosphate buffer with or without the presence of MT. The solution was immediately mixed after addition of SIN-1 or ONOO− because under these conditions ONOO− spontaneously decays with a half-life of less than 2 s. The reaction solution (30 μl) was mixed with 3 μl of electrophoresis loading buffer and loaded onto a 1% agarose gel prepared in TAE buffer (40 mM Tris species; RNS, reactive nitrogen species; ONOO−, peroxynitrite; SIN-1, 3-morpholinosydnonimine; TBARS, thiobarbituric acid-reactive substance; LDL, low density lipoprotein; SOD, superoxide dismutase; SC, supercoiled; OC, open circle; NO, nitric oxide.

This paper is available on line at http://www.jbc.org

Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 50, Issue of December 15, pp. 38957–38960, 2000
Published, JBC Papers in Press, October 20, 2000, DOI 10.1074/jbc.C000593200
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**RESULTS**

**MT Protection from ONOO\(^{-}\)-induced DNA Damage**—When pTZ 18U plasmid DNA was exposed to SIN-1, the native SC DNA was converted to a relaxed OC DNA with single strand breaks (Fig. 1A). This effect of SIN-1 was dose-dependent (Fig. 1B). Although this damage was also time-dependent, it reached a maximum damage level within 3 h of exposure to SIN-1 at 37 °C (Fig. 1, A and C). In the presence of MT, the extent of SIN-1-mediated DNA damage decreased in a MT dose-dependent manner (Fig. 1, D and E). Because SIN-1 produces NO and O\(_2\)\(^{\cdot}\) simultaneously in physiological solution at 37 °C and the reaction between NO and O\(_2\)\(^{\cdot}\) leads to the formation of ONOO\(^{-}\), DNA damage by SIN-1 is thus assumed to be the effect of ONOO\(^{-}\). To explore the possibility that MT prevents SIN-1-induced DNA damage through the reaction between MT and ONOO\(^{-}\), synthesized ONOO\(^{-}\) was added directly to the reaction system. As shown in Fig. 2A, ONOO\(^{-}\) is able to induce DNA damage in a dose-dependent manner, and MT conveyed significant protection from this damage (Fig. 2B). This suggests that MT is a potent scavenger of the ONOO\(^{-}\) radical. To further determine the specificity of MT reaction with ONOO\(^{-}\), the effect of SOD on DNA damage induced by SIN-1 and ONOO\(^{-}\) was examined. As shown in Fig. 3, SOD (12.5 units in 30-μl reaction system, which equals about 4.0 μM SOD) is able to prevent DNA damage caused by 0.1 mM SIN-1, but not by ONOO\(^{-}\).

**MT Protection from SIN-1- or ONOO\(^{-}\)-dependent LDL Oxidation**—When LDL was incubated for 18 h at 37 °C with SIN-1 (1.0 mM), lipid peroxidation was detected by the TBARS assay. The same oxidative damaging effect of the synthesized ONOO\(^{-}\) on LDL was also detected. Both SIN-1 and synthesized ONOO\(^{-}\) induced a dose-dependent oxidative injury to LDL (Fig. 4A). MT significantly protected LDL from peroxidation by SIN-1 or ONOO\(^{-}\) (Fig. 4B). The TBARS result was confirmed by a lipid hydroperoxide assay (Cayman Chemical, Ann Arbor, MI), and the same protective effect of MT was observed (data not shown).

**DISCUSSION**

In the present study, we provide, for the first time, direct evidence that MT can react with ONOO\(^{-}\) to protect DNA and lipoprotein from oxidative damage. This further extends our understanding of the characteristics of MT antioxidant action. The reaction of MT with ONOO\(^{-}\) is particularly important in preventing oxidative tissue injury because ONOO\(^{-}\) has been shown to be highly responsible for pathogenesis under a diversity of disease conditions. These include inflammatory and neurodegenerative disease, myocardial dysfunction, and environmental toxicity (19, 20, 24).

The role of ONOO\(^{-}\) in pathogenesis has been demonstrated to be associated with its interaction with the MAPK signaling pathway. Exposure of cultured cells to SIN-1 or directly to ONOO\(^{-}\) caused immediate activation of p38 MAPK with the induction of apoptotic cell death, suggesting the role of ONOO\(^{-}\) in activating signaling transduction pathways capable of inducing apoptosis (9–11). However, an inhibitor of p38 MAPK, SB202190, only partially reduced the activation of caspase-3 and apoptotic cell death caused by ONOO\(^{-}\) (10). This suggests that the activation of p38 MAPK is only one of the pathways induced by ONOO\(^{-}\) for the activation of caspase-3 and induction of apoptosis. Other pathways leading to caspase-3 activation and apoptosis may involve in ONOO\(^{-}\)-induced pathogenesis. Recent studies have shown that ONOO\(^{-}\) causes mitochondrial structural and functional alterations through lipid peroxidation and protein sulfhydryl oxidation (12–14). These changes lead to cytochrome c release from mitochondria. This in turn results in activation of caspase-3 through activated caspase-9 by cytochrome c. In addition, it has been shown
that ONOO$^-$ directly reacts with cytochrome c in a cell-free system (14).

In our recent studies, we have demonstrated that MT prevents DOX-induced cardiomyocyte apoptosis through inhibition of p38 MAPK activation (17). We also have observed that MT inhibited mitochondrial cytochrome c release induced by DOX.$^2$ It has been well known that DOX generates ROS and RNS in the myocardium. However, the species of ROS or RNS responsible for the pathogenesis has not been known until recently. DOX binds to the endothelial isoform of nitric-oxide synthase and undergoes endothelial nitric-oxide synthase-mediated reduction to become the semiquinone radical (15). This leads to generation of ONOO$^-$ and H$_2$O$_2$. Furthermore, the hypothesis that DOX-induced cardiac toxicity is associated with the accumulation of ONOO$^-$ formation has been proven in the intact animals treated with DOX (16).

The present study has an important implication of understanding the mechanism of action of MT in preventing DOX-induced cardiotoxicity. The induction of apoptotic cell death is an important mechanism of cardiotoxicity, and the formation of ONOO$^-$ plays a critical role in this mechanism. In particular, the protection by MT against DOX-induced apoptosis in cardiomyocytes has been shown to be associated with inhibition of both p38 MAPK activation and mitochondrial cytochrome c release. MT may react with ONOO$^-$ to block the activation of p38 MAPK and the release of mitochondrial cytochrome c. This consequently leads to the protection by MT against DOX-induced apoptosis in mouse myocardium in vivo and cardiomyocytes in vitro.

In this study, we observed that ONOO$^-$-induced DNA damage was protected by MT, but the protection was not directly dose-dependent on MT concentrations (Fig. 2B). It is possible that the kinetics of reactions of ONOO$^-$ with MT and DNA define the protective effect of MT rather than its concentration because ONOO$^-$ is a very short-lived species in solution ($t_{1/2} \approx 2$ s). Another explanation is the use of commercial MT, which may contain a limited amount of copper. Copper is a transition metal that generates HO$^+$ radical in the presence of reductant such as ascorbate and O$_2^-$ through the Fenton reaction. DNA is a very sensitive target of HO$^+$ (25). The fact that commercial MT contains copper and that RNS can react with MT to release metals from MT have been demonstrated (26). To explore this possibility in the present study, we have monitored the absorbance at 480 nm in the presence of bathocuproinedisulfonic acid along with MT, ONOO$^-$, and H$_2$O$_2$. If copper is released from MT, a complex of cuprous with bathocuproinedisulfonic acid will be formed (25) and the increase in the absorbance in proportion to MT concentrations will be observed. This phenomenon was indeed apparent (data not shown). Therefore, a balance between MT protection and more DNA damage in proportion to MT concentrations will be observed. This phenomenon is likely to understand the characteristics of MT protection against ONOO$^-$-induced DNA damage.
MT Inhibits ONOO$^-$-induced DNA and Lipoprotein Damage

damage, the present study provides the first evidence that MT protects from ONOO$^-$-induced DNA and protein damage.

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