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N,N'-Dimethylthiourea dioxide formation from *N,N'*-dimethylthiourea reflects hydrogen peroxide concentrations in simple biological systems

(O_2 metabolites/lung injury/oxidants/antioxidants)

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ABSTRACT **We hypothesized that measurement of a specific product from reaction of *N,N'*-dimethylthiourea (Me_2TU) and H_2O_2 would provide a good indication of the H_2O_2 scavenging and protection seen after addition of Me_2TU to biological systems. We found that addition of H_2O_2 to Me_2TU yielded a single stable product, Me_2TU dioxide. Me_2TU dioxide formation correlated with Me_2TU consumption as a function of added H_2O_2 concentration and was prevented by simultaneous addition of catalase (but not boiled catalase), superoxide dismutase, dimethyl sulfoxide, mannitol, or sodium benzoate. Me_2TU dioxide formation, Me_2TU consumption, and H_2O_2 concentration increases occurred in mixtures containing phorbol 12-myristate 13-acetate (PMA) and normal human neutrophils but not in mixtures containing PMA and neutrophils from patients with chronic granulomatous disease or in mixtures containing PMA and normal neutrophils and catalase. Me_2TU dioxide formation also occurred in isolated rat lungs perfused with Me_2TU and H_2O_2 but not in lungs perfused with Me_2TU and elastase, histamine, or oleic acid. In contrast, Me_2TU dioxide formation did not occur after exposure of Me_2TU to ^{60}Co-generated hydroxyl radical or hypochlorous acid in the presence of catalase. The results indicate that reaction of Me_2TU with H_2O_2 selectively forms Me_2TU dioxide and that measuring Me_2TU dioxide formation from Me_2TU may be useful for assessing the presence and significance of H_2O_2 in biological systems.**

Toxic oxygen metabolites appear to participate in a multitude of normal and abnormal biological responses (1–6). Because O_2 metabolites are highly reactive and present in low concentrations, it has been difficult to ascertain their contribution in biological systems (2, 7). The contribution of toxic O_2 metabolites is usually assessed in biological systems by observing changes after addition of agents that decrease O_2 metabolite concentrations *in vitro*. One of these agents is *N,N'*-dimethylthiourea (Me_2TU), a highly permeant molecule, which decreases injury in a wide variety of biological systems (8–20). However, because Me_2TU can scavenge hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), or hypochlorous acid (HOCl) *in vitro*, its mechanism of action in biological systems is often questioned (8–13, 21). Recently, we addressed this concern by showing that Me_2TU decreased after reaction with H_2O_2 *in vitro* and then used Me_2TU consumption to assess H_2O_2 and H_2O_2-dependent processes in simple biological systems (8). Me_2TU consumption occurred as a function of added amounts of H_2O_2 and in proportion to corresponding degrees of H_2O_2-mediated injury in endothelial cells and isolated perfused lungs and kidneys (9–11). However, since Me_2TU might also be consumed by reaction with either ·OH or HOCl (8, 21), we subsequently reasoned that increased specificity or sensitivity in detecting the small concentrations of H_2O_2 that probably occur in physiological systems would be achieved if a specific product from reaction of H_2O_2 and Me_2TU could be identified and measured in biological systems.

METHODS

Purification and Determination of Me_2TU Dioxide. H_2O_2 (J. T. Baker Chemical, Phillipsburg, NJ) was added to triply distilled H_2O and Me_2TU (Alfa–Ventron, Danvers, MA) and allowed to react for 60 min in an ice bath. The product was purified by using a Waters Resolve C_{18} 5-μm semipreparative column (7.8 mm × 30 cm) and lyophilized. Elemental analysis (by Huffman Laboratories, Denver, CO), IR, NMR, and mass spectroscopy analyses were conducted on the purified product.

Measurement of Me_2TU or Me_2TU Dioxide Concentrations by Gas Chromatography (GC) or High-Pressure Liquid Chromatography (HPLC). An internal standard (1%) for GC analysis was made by dissolving diethyl sulfone (3.5 g, ICN Pharmaceutical, Plainview, NJ) in deionized H_2O (100 ml). In some experiments, catalase (Worthington), urea (Fisher Scientific, Fairlawn, NJ), thiourea (J. T. Baker Chemical), HOCl, sodium benzoate, mannitol, superoxide dismutase (SOD), dimethyl sulfoxide (Me_2SO), or 1,3-dimethylurea (all from Sigma) was added to samples of H_2O_2 or Me_2TU. Me_2TU and Me_2TU dioxide concentrations were assessed by using a GC instrument, equipped with a free induction decay detector and integrator (8). The integrator was programmed to print out peak areas and retention times. The peak area for Me_2TU dioxide was divided by the peak area for the internal standard to give a peak area ratio. Incubations were conducted in triply distilled H_2O or potassium phosphate buffer (0.05 M, pH 7.4). Me_2TU dioxide concentrations also were measured by using HPLC analysis (Waters model 510 pump) with a Resolve C_{18} 5-μ reverse-phase column (3.9 mm × 15 cm) with H_2O as the isocratic mobile phase at a flow rate of 1 ml/min. The effluent was monitored at 229 nm with a spectrophotometric detector, with diluted Me_2SO used as an internal standard for HPLC analysis.

Isolation of Neutrophils. Neutrophils were isolated from heparinized (20 units/ml) venous blood collected from healthy medication-free adult donors and an individual with X chromosome-linked chronic granulomatous disease (CGD) by using hetastarch (Hespan 6%; DuPont, Waukegan, IL)

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Abbreviations: Me_2TU, dimethylthiourea; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; CGD, chronic granulomatous disease.

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sedimentation and Percoll gradient separation and erythrocyte lysis (22, 23). Neutrophils were counted in a hemocytometer and resuspended in complete Hanks' balanced salt solution (HBSS, GIBCO, Chagrin Falls, OH). Phorbol 12-myristate 13-acetate (PMA, Sigma) was dissolved in Me_2SO and stored at −70°C (23).

Measurement of H_2O_2 Concentrations. Samples were acidified with 50% trichloroacetic acid (Sigma, 0.1 ml) and centrifuged. Supernatants (0.2 ml) were combined with isotonic saline (0.8 ml), ferrous ammonium sulfate (10 μM, 0.2 ml), and potassium thiocyanate (2.5 M, 0.1 ml) and then assayed spectrophotometrically at 480 nm (24).

Exposure to Irradiation. Samples were exposed to ^{60}Co γ-irradiation under N_2O. Characteristic EPR and other values were obtained, confirming ·OH production (25).

Measurement of Acute Edematous Injury in Isolated Perfused Rat Lungs. Rats were anesthetized with pentobarbital (100 mg/kg i.p.) (26). After tracheostomy, rats were continuously ventilated with a warmed humidified mixture of 95% room air and 5% CO_2. The thorax and abdomen were then opened with a ventral midline incision and heparin (150 units) was injected. Cannulae were placed in the pulmonary artery and left atrium. The lung vasculature was perfused clear with modified Krebs/Henseleit solution that contained Ficoll-70 (Pharmacia). Heart and lungs were then removed, suspended by the trachea, and weighed by using a force (weight) displacement transducer. Pulmonary artery perfusion pressures were measured by pressure transducer through a cannula placed in the pulmonary artery.

Statistical Analyses. Data were analyzed by using analysis of variance (ANOVA) with Student–Newman–Keuls (SNK) tests (27).

RESULTS

Identification of Me_2TU Dioxide as the Product from Reaction of Me_2TU and H_2O_2 *in Vitro*. Reaction of Me_2TU and H_2O_2 yielded a single peak on HPLC (Fig. 1) or GC (data not shown) tracings (28, 29). Elemental analyses revealed that the product from reaction of Me_2TU with H_2O_2 fit the predicted formula $C_3H_8N_2O_2S$ for Me_2TU dioxide. Calculated: C, 26.46%; H, 5.92%; N, 20.57%; O, 23.50%; S, 23.54%. Observed: C, 26.48%; H, 5.99%; N, 20.48%; O, 23.59%; S, 23.25%.

In addition, IR spectroscopy showed the typical sulfinic acid (S=O) absorption band in the region of 1090 cm^{-1} (30). ^{13}C NMR showed three distinct carbons with hindered rotation about the C—N bond (31). Electron impact mass spectrometry revealed that the product sample fragmented into two peaks representing sulfur dioxide and the corresponding formamidine (32). Negative chemical ionization spectra showed a (M − 1) ion for the sulfinic acid at m/z 135. Positive fast atom bombardment spectra demonstrated a confirmatory (M + 1) ion at m/z 137. The reaction rate of Me_2TU with H_2O_2 *in vitro* was calculated to be 1.4 $M^{-1}\cdot min^{-1}$ (Fig. 2). Me_2TU dioxide decomposed in H_2O at room temperature but not in H_2O or potassium phosphate buffer kept at 0°C for 2 weeks (data not shown). Me_2TU dioxide was also stable during incubation for 30 min at 37°C with H_2O_2, and for 60 min when incubated at 37°C with neutrophils or erythrocytes (data not shown). Concentrations of Me_2TU (500 nM to 1 mM) or Me_2TU dioxide (700 nM to 1 mM) were measurable by GC or HPLC analyses, respectively.

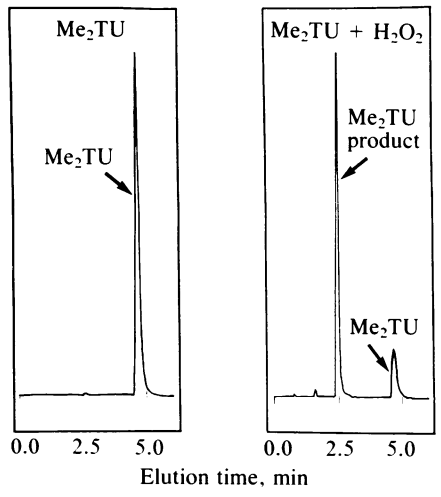

FIG. 1. Characteristic tracings from HPLC analysis of Me_2TU or a mixture of Me_2TU with H_2O_2 *in vitro*.

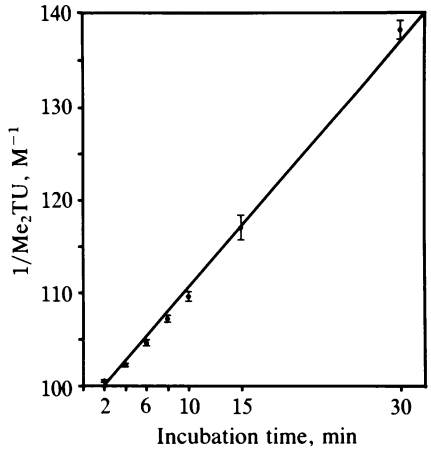

FIG. 2. Determination of second-order rate for reaction of Me_2TU with H_2O_2 *in vitro*. Initial concentrations (C_0) of the two reactants were equal. The equation used to calculate the rate was $1/C - 1/C_0 = kt$, in which C is concentration and t is time. Each value is the mean ± 1 SEM of four determinations.

Specificity of Me_2TU Dioxide Formation After Reaction of H_2O_2 with Me_2TU *in Vitro*. Addition of increasing concentrations of H_2O_2 caused linear decreases in Me_2TU concentrations (Me_2TU consumption) and corresponding linear increases in Me_2TU dioxide concentrations (Me_2TU dioxide formation) in buffered solutions (GC analysis, Fig. 3) or unbuffered solutions (data not shown). Less Me_2TU dioxide formation occurred after addition of catalase but not boiled catalase, SOD, Me_2SO, mannitol, or sodium benzoate to mixtures of H_2O_2 and Me_2TU *in vitro* (Table 1).

Me_2TU dioxide formation did not occur after reaction of Me_2TU with ·OH or HOCl in the presence of catalase *in vitro*.

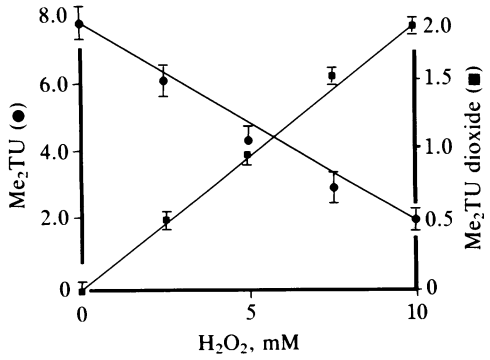

FIG. 3. Me_2TU concentration decreases as Me_2TU dioxide concentration increases as a function of added H_2O_2 concentration *in vitro*. Me_2TU and Me_2TU dioxide concentrations were measured by GC and are expressed as peak area relative to area of the internal standard peak. Each value is the mean ± 1 SEM of three determinations.

Table 1. Effect of O_2 metabolite scavengers on Me_2TU dioxide concentrations in mixtures containing Me_2TU and H_2O_2 *in vitro*

Additions	Me_2TU dioxide, mM	
	Unbuffered medium	Buffered medium
None	0 (3)	0 (3)
H_2O_2 (36 mM)	3.5 ± 0.6* (3)	4.6 ± 0.2* (3)
H_2O_2 + catalase (250 μg/ml)	0† (3)	0† (3)
H_2O_2 + boiled catalase	4.3 ± 0.3* (3)	4.2 ± 0.5* (3)
H_2O_2 + SOD (125 μg/ml)	4.2 ± 0.3* (3)	3.0 ± 0.5* (3)
H_2O_2 + Me_2SO (12.3 mM)	3.9 ± 0.5* (3)	3.5 ± 0.3* (3)
H_2O_2 + mannitol (12.5 mM)	3.1 ± 0.5* (3)	3.3 ± 0.4* (3)
H_2O_2 + Na benzoate (12.5 mM)	3.7 ± 0.4* (3)	4.9 ± 0.7* (3)
H_2O_2 + albumin (2.5 mg/ml)	3.0 ± 0.3* (3)	3.6 ± 0.1* (3)

Me_2TU (50 mM) and the substances in the first column were mixed and then incubated for 30 min at 37°C. Results are presented as mean ± SEM, with number of determinations in parentheses.
*Value significantly different ($P < 0.05$) from value obtained after no additions.
†Value significantly different ($P < 0.05$) from value obtained after addition of H_2O_2.

Irradiation (^{60}Co) under N_2O for 10 min caused Me_2TU consumption (14 ± 2% of initial value, $n = 5$), Me_2TU dioxide formation (3.4 ± 1.0 μM, $n = 5$), and H_2O_2 production (2.6 ± 2.6 μM, $n = 5$). However, when catalase was added, irradiation caused reduced Me_2TU consumption (6 ± 2% of initial value, $n = 5$) but not Me_2TU dioxide formation (0.0 ± 0.0 μM, $n = 5$) or H_2O_2 production (0.0 ± 0.0 μM, $n = 5$). Thus, reaction of Me_2TU with either H_2O_2 or ·OH consumes Me_2TU but only reaction of Me_2TU with H_2O_2 (and not ·OH) yields Me_2TU dioxide. Addition of HOCl and catalase also did not produce Me_2TU dioxide (0 ± 0 μM, $n = 5$) from Me_2TU *in vitro*. Me_2TU dioxide also was not measurable in mixtures containing H_2O_2 and thiourea, urea, or dimethylurea, in mixtures of Me_2TU in H_2O or phosphate buffer without H_2O_2, or in mixtures of H_2O_2 with the other constituents without Me_2TU (data not shown).

Me_2TU Dioxide Concentrations in Mixtures Containing Human Neutrophils. Me_2TU dioxide concentrations, Me_2TU consumption, and H_2O_2 concentrations were increased in mixtures containing neutrophils and PMA compared to mixtures containing neutrophils alone (Table 2). Addition of catalase (but not SOD) decreased Me_2TU dioxide concentrations, Me_2TU consumption, and H_2O_2 concentrations in mixtures containing normal human neutrophils and PMA. By comparison, Me_2TU dioxide formation, Me_2TU consumption, and H_2O_2 concentration increases did not occur in mixtures containing CGD neutrophils and PMA (Table 2).

Me_2TU Dioxide Concentrations in Isolated Lungs Perfused with H_2O_2. Addition of H_2O_2 caused Me_2TU dioxide formation and acute edematous injury (increases in lung weight gains and pulmonary artery perfusion pressures) in isolated lungs perfused with Me_2TU; Me_2TU dioxide formation and injury did not occur in isolated lungs of rats perfused only with buffer and Me_2TU without added H_2O_2 (Table 3). In contrast to H_2O_2, addition of elastase, histamine, or oleic acid to perfusates of isolated lungs containing Me_2TU did not produce Me_2TU dioxide regardless of the development of acute edematous lung injury.

DISCUSSION

We found that reaction of Me_2TU and H_2O_2 yields Me_2TU dioxide and that Me_2TU dioxide formation from Me_2TU can be used to detect H_2O_2 in simple biological systems. Reaction of Me_2TU with H_2O_2 yielded Me_2TU dioxide as a single stable product. This Me_2TU dioxide formation was inhibited by catalase and did not occur after reaction of Me_2TU with ·OH or HOCl *in vitro* in the presence of catalase. Me_2TU dioxide formation from Me_2TU also occurred with stimulated normal neutrophils (but not neutrophils from CGD patients) in proportion to the amounts of generated H_2O_2 and consumed Me_2TU. Moreover, prior addition of catalase to mixtures containing normal neutrophils and PMA decreased Me_2TU dioxide formation as well as corresponding H_2O_2 concentration increases and Me_2TU consumption. Me_2TU dioxide formation also occurred in perfusates of isolated lungs perfused with H_2O_2 and Me_2TU, but not in isolated lungs perfused with Me_2TU and histamine, elastase, or oleic acid, regardless of the development of acute edematous injury.

There is considerable interest in Me_2TU because Me_2TU treatment dramatically decreases injury in a number of biological models in which damage appears to involve O_2 metabolites (9–11). Me_2TU protects alveolar macrophages (12) and lungs (13) from hyperoxic damage, reduces acute edematous lung injury in rabbits treated with PMA (14), and decreases injury in isolated lungs or kidneys perfused with neutrophils and PMA or chemically generated O_2 metabolites (9–11, 15). Me_2TU treatment also effectively reduces cerebral, renal, and myocardial damage after ischemia–reperfusion insults (16–20). In each instance, Me_2TU has been assumed to protect by scavenging O_2 metabolites, in large part, because addition of Me_2TU decreases O_2 metabolite concentrations *in vitro*. However, better ways are needed to assess the nature of the protective effects of Me_2TU in biological systems (2, 7).

Although the abilities of Me_2TU to scavenge O_2 metabolites are well recognized *in vitro* and suspected *in vivo*, some controversy exists regarding whether Me_2TU protects by specifically inactivating H_2O_2, ·OH, or HOCl in biological systems. In simple nonbiological systems, Me_2TU can react with H_2O_2, ·OH, or HOCl (8, 21). The present study emphasizes this point by showing that Me_2TU consumption occurs after reaction with either H_2O_2 or ·OH *in vitro*. Others have

Table 2. Me_2TU dioxide, Me_2TU, and H_2O_2 concentrations in mixtures of normal (NL) and CGD human neutrophils (PMN) and PMA *in vitro*

Additions	Me_2TU dioxide, μM	Me_2TU, mM	H_2O_2, μM
NL PMN	0.3 ± 0.3 (6)	0.31 ± 0.05 (4)	0.5 ± 0.3 (20)
NL PMN + PMA	13.2 ± 1.5* (6)	0.19 ± 0.02* (3)	98.9 ± 6.4* (10)
NL PMN + PMA + catalase	6.9 ± 1.6* (6)	0.27 ± 0.02† (3)	0.5 ± 0.3† (5)
NL PMN + PMA + SOD	16.9 ± 2.7* (6)	0.21 ± 0.03* (3)	104.9 ± 8.2* (5)
CGD PMN + PMA	0† (4)	0.32† (2)	0† (2)
CGD PMN	0† (4)	0.30† (2)	0† (2)

Me_2TU (1 mM) and the cells and substances listed in the first column were incubated for 60 min at 37°C. PMA was added at 1 μg/ml and catalase or SOD at 100 μg/ml. Me_2TU dioxide generation, Me_2TU consumption, and H_2O_2 generation were measured with 2×10^7, 1×10^7, and 1×10^6 PMN per ml, respectively. Results are mean ± SEM, with number of determinations in parentheses.
*Value significantly different ($P < 0.05$) from value obtained for normal neutrophils.
†Value significantly different ($P < 0.05$) from value obtained for normal neutrophils + PMA.

Table 3. Me_2TU dioxide concentrations in perfusates, weight gains, and pulmonary artery pressure (PAP) increases of isolated rat lungs perfused with Me_2TU and H_2O_2, elastase, histamine, or oleic acid

Additions	Me_2TU dioxide, μM	Weight gain, g	PAP increase, mm Hg
None	0 (8)	1.5 ± 0.2 (8)	3.8 ± 0.5 (8)
H_2O_2 (10 mM)	24 ± 0.7* (3)	15.0 ± 2.3* (3)	11.2 ± 4.6* (3)
Elastase (7 mg)	0 (2)	15.0* (2)	8.5* (2)
Histamine (1.4 mg)	0 (2)	0 (2)	0.3* (2)
Oleic acid (250 mg)	0 (2)	10.2* (2)	28.0* (2)

Perfusates contained 14 mM Me_2TU and the additions in the first column. Results are mean ± SEM, with number of determinations in parentheses; 1 mm Hg = 133 Pa.
*Value significantly different ($P < 0.05$) from value obtained with no additions.

shown that HOCl can cause Me_2TU consumption *in vitro* (21). However, only reaction of Me_2TU with H_2O_2 yields Me_2TU dioxide; in the presence of catalase, reaction of Me_2TU with ·OH or HOCl does not yield Me_2TU dioxide. Thus, finding Me_2TU dioxide indicates that Me_2TU has reacted with H_2O_2, regardless of whether or not it has also reacted with some other agent.

Because ·OH and HOCl are so much more reactive than H_2O_2, and as a result, may react indiscriminately and rapidly with various biological molecules, it should be much easier to scavenge the less reactive H_2O_2 (33–35). Accordingly, even though Me_2TU may react much more rapidly with ·OH or HOCl than with H_2O_2 *in vitro*, the situation may be quite different in biological systems, where accessibility of Me_2TU to unreacted H_2O_2 may favor reaction of Me_2TU with H_2O_2 rather than highly reactive ·OH or HOCl. The latter is suggested because (except for irradiation), ·OH or HOCl (via myeloperoxidase) theoretically is formed only from H_2O_2 in biological systems. Moreover, addition of Me_2TU can inhibit H_2O_2- and Fe^{2+}/EDTA-mediated Me_2SO consumption *in vitro* (32). This conclusion is also supported by our observations that Me_2TU dioxide is made in biological situations involving neutrophils and isolated lungs. In addition, treatment with Me_2TU (but not urea) decreases injury and H_2O_2-dependent inactivation of catalase by aminotriazole in brains of gerbils subjected to ischemia–reperfusion insults (17). Production of Me_2TU dioxide from Me_2TU may be useful in determining the mechanism of protection achieved after addition of Me_2TU and in distinguishing reactions with H_2O_2 from those with ·OH, HOCl, or other constituents in biological systems. In addition, measurement of decreases in injury and corresponding increases in Me_2TU dioxide concentrations could help determine if treatment with agents that purportedly prevent injury by decreasing H_2O_2 concentrations do so by decreasing H_2O_2 or H_2O_2-dependent reactions or by altering some other non-H_2O_2-dependent mechanism. We hope that this method or this principle will prove applicable to intact animal models, and eventually patients, in which increased amounts of O_2 metabolites are purportedly being generated endogenously (2, 7).

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