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Human Hydroxysteroid Sulfotransferase 2A1 is Down Regulated by Nitric Oxide in Human Hep G2 Cells

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Abstract: Nitric oxide is known to regulate gene expression by modulating the activation of transcription factors. In organisms, it is synthesized from l-arginine in the presence of an enzyme called nitric oxide synthase and it can act as a biosignaling molecule. Sulfotransferases (SULTs) are important phase II drug-metabolizing enzymes responsible for drug metabolism and xenobiotics detoxification. SULTs are also important in the regulation and metabolism of endogenous hormones and other important signaling molecules. This report investigates human SULT gene regulation by nitric oxide and the potential molecular mechanisms. Here we report the effect of nitric oxide on the expression of human SULT1A1 and SULT2A1, the two major human SULTs. When Hep G2 cells were treated with diethylene triamine/nitric oxide (DETA/NO), Western blot and q-RT-PCR data suggested that SULT2A1 was downregulated, while SULT1A1 was not significantly affected. The downregulation of SULT2A1 occurred in a time- and concentration-dependent manner. DETA/NO induced the phosphorylation of extracellular signal-regulated protein kinase (ERK). PD98059, a pharmacological inhibitor of ERK, prevented the DETA/NO downregulation of SULT2A1. The present study suggested that the downregulation of SULT2A1 involves ERK cell signaling pathways. This is the first report on nitrative stress regulation of human SULT2A1. The results are important for understanding biological functions of SULTs in organisms under stress.

Key words: Sulfation, phase II drug metabolizing enzymes, nitrosative stress, gene regulation, mitogen-activated protein kinases

INTRODUCTION

Sulfotransferases (SULTs) are one of the major families of phase II drug-metabolizing enzymes (Gamage *et al*., 2006; Runge-Morris and Kocarek, 2009). They catalyze the sulfation of hydroxyl-containing compounds. The co-substrate for sulfation for all SULTs is adenosine 3'-phosphate 5'-phosphosulfate (PAPS). The substrate specificities of some SULTs are very broad. Most hydroxyl-containing compounds (phenols and alcohols) are substrates for SULT isoforms.

Sulfation is widely observed in various biological processes (Nimmagadda *et al*., 2006; Harris and Waring, 2008). The biological activities of various biological signaling molecules-including hydroxysteroid hormones, glucocorticoid hormones, thyroid hormones, neurotransmitters, heparan, peptides and proteins-can be altered through sulfation (Nimmagadda *et al*., 2006; Lindsay *et al*., 2008). Sulfation usually leads to the inactivation of biological signaling molecules, as the sulfated forms are usually unable to bind to receptors (Coughtrie *et al*., 1998; Runge-Morris and Kocarek, 2005; Nimmagadda *et al*., 2006). SULT-catalyzed sulfation regulates the biological activities and functions of many biological signaling molecules (Harris and Waring, 2008).

SULTs also catalyze the sulfation of a broad range of xenobiotics (Glatt and Meinl, 2004). Sulfation of xenobiotics is mainly associated with detoxification: biotransformation of a relatively hydrophobic xenobiotic compound into a more water-soluble sulfate ester that is readily excreted (Nimmagadda *et al*., 2006). However, there are important exceptions wherein the formation of chemically reactive sulfate esters is an essential step in the metabolic pathways leading to carcinogenic responses (Falany and Wilborn, 1994; Runge-Morris and Kocarek, 2005). Most sulfation products are stable enough for excretion, while other sulfate ester products are reactive toward nucleophilic sites on DNA, RNA and protein and thus become involved in the initiation of carcinogenesis and other toxic responses.

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It is known that hormones and xenobiotics regulate SULT expression (Runge-Morris *et al*., 1998; Seo *et al*., 2007). Improper regulation of SULTs by xenobiotics and other factors can cause biosignaling molecules to function improperly and may cause cancer and other diseases (Hengstler *et al*., 1998; Glatt and Meinl, 2004; Lindsay *et al*., 2008). Induction of SULTs increases xenobiotic detoxification, but at the same time, SULT induction can also cause drug resistance. Increased levels of SULTs can also increase the bioactivation of procarcinogens and promutagens (Coughtrie *et al*., 1998).

Nitric Oxide (NO) is a biosignaling molecule involved in a variety of physiological functions such as vasodilation, fertilization, differentiation, inflammation and apoptosis (Hemish *et al*., 2003). NO is known to regulate gene expression by modulating the activation of transcription factors or by mediating the stability of mRNA (Dhakshinamoorthy and Porter, 2004). Superoxide dismutase (SOD), catalase and some other antioxidant enzymes are transcriptionally upregulated by NO in neuroblastoma cells (Dhakshinamoorthy and Porter, 2004). The role of NO in modulating the function of some transcription factors, such as HNF-3, TTF-1, NF-kB, AP-1 and VDR/RXR, has been documented (Pilz *et al*., 1995; Matthews *et al*., 1996; Kimura *et al*., 2000; Kroncke and Carlberg, 2000; Lee *et al*., 2008).

NO also plays an important role in the regulation of surfactant protein-B and interleukin-8 genes in lung; surfactant protein-B and interleukin-8 are important mediators of lung stability and inflammation, respectively (Salinas *et al*., 2003; Sparkman and Boggaram, 2004). Kinases in cell signaling pathways are activated by diverse extracellular stimuli, such as ultraviolet irradiation, heat shock, osmotic stress and many other types of oxidative stress. However, not all kinases are activated by the same stress. These kinases modulate gene function and some of the transcription factors as well (Yeh and Yen, 2006). Mitogen-activated protein kinases (MAPKs) belong to the family of serine/threonine kinases and play an important role in coordinating various extracellular signals to different biological functions, such as gene expression, cell proliferation, differentiation and cell death (Mokhtari *et al*., 2008).

Very few studies have investigated the nitrosative stress regulation of SULTs. Dr. Charles Strott's laboratory has reported the down-regulation of rat steroid SULT2B1a by NO of both endogenous and exogenous origin using rat C6 glioma cell line (Kohjitani *et al*., 2008). The present study reports that NO downregulated SULT2A1 in human Hep G2 cells. SULT1A1, however, was not significantly affected. Regulation of SULT2A1 involves the activation of the extracellular signal-regulated protein kinase (ERK) cell signaling pathway.

MATERIALS AND METHODS

Materials. Adenosine 3'-phosphate 5'-phosphosulfate (PAPS), Dulbecco's Modified Eagle's Medium (DMEM) cell culture media, 0.25% trypsin-EDTA and diethylene triamine/nitric oxide adduct (DETA/NO) were purchased from Sigma (St. Louis, MO). SDS-polyacrylamide gel electrophoresis reagents and protein assay reagents were obtained from Bio-Rad (Hercules, CA). Western blot chemiluminescence reagent kits (Super Signal West Pico Luminol/Enhancer solutions) were purchased from Thermo Scientific (Rockford, IL). PVDF membranes used for Western blotting analyses were purchased from Millipore Corporation (Bedford, MA). Rabbit anti-hSULT2A1 and anti-hSULT1A1 antibodies were purchased from Biovision (Mountain View, CA). Phospho-ERK and total-ERK were supplied by Cell Signaling Technology, Inc. (Beverly, MA). Fetal bovine serum used in the cell media was obtained from Hyclone (Logan, UT). [1,2,6,7-^{3}H(*N*)] dehydroepiandrosterone ([3H]DHEA, 60 Ci/mmol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). This research project was conducted at the Enzymology and Toxicology Laboratory (Guangping Chen), Department of Physiological Sciences, Oklahoma State University between 2008 and 2009.

Cell culture: Hep G2 cells (a human hepatocarcinoma cell line, ATCC number: HB-8065™) obtained from American Type Culture Collection (Manassas, VA) were grown in DMEM containing 10% fetal bovine serum. Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2. The medium was replaced every 3 days.

Preparation of cell lysates: Hep G2 cells grown in control media or media containing DETA/NO were washed twice with cold phosphate-buffered saline and incubated in lysis buffer (50 mM Tris-Cl [pH 7.5] containing 250 mM sucrose, 10 μg mL^{-1} trypsin inhibitor and 10 μg mL^{-1} PMSF) for 15 min on ice. Cell lysates were cleared by centrifugation at 13,000 rpm for 15 min and the supernatant was used for Western blotting and enzyme activity assays, as previously described (Chen *et al*., 2005; Maiti *et al*., 2005).

Immunoblot analysis: SDS-PAGE separation and transfer of proteins onto membranes were carried out with a Mini Trans-Blot cell apparatus (Bio-Rad), according to the

manufacturer's instructions. Equal amounts of protein were separated by SDS-PAGE on 10% Bis-Tris gels and electrophoretically transferred onto PVDF membranes. Membranes were successively incubated with primary polyclonal antibodies against hSULT2A1, hSULT1A1, phospho-ERK and total-ERK at 1:1000 overnight at 4°C, followed by HRP-conjugated goat anti-rabbit secondary antibody (Pierce Biotechnology, Rockford, IL) at 1:2000 for 1 h at room temperature. Protein bands were visualized by the Enhanced Chemiluminescence (ECL) detection method (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. Fluorescent bands were developed with 3 mL of substrate containing the same volume each of Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico Stable Peroxidase solution for 5 min at room temperature. The fluorescent band signals were recorded with a Bio-Rad Versa Doc 5000 Image System and densitometry analysis was performed with Quantity One software (Bio-Rad, Hercules, CA).

Enzyme activity assay: Dehydroepiandrosterone (DHEA) sulfation activities in cell lysates were determined by the radioactive assay method, as described previously (Chen *et al*., 2003; Maiti and Chen, 2003a, b). Briefly, 50 μg of protein from the cell lysates were used as the enzyme source for each assay. [^{3}H]-DHEA (diluted to 0.4 Ci mmol^{-1}; 2 μM final concentration) was used as a substrate. For all assays, 20 μM PAPS was used. All enzymatic assays were performed in a total reaction volume of 250 μL. After 30 min of incubation at 37°C in a shaking water bath, the reaction was stopped by adding 250 μL of stop buffer (0.25 M Tris, pH 8.7). Extraction was performed twice by adding 0.5 mL of water-saturated chloroform. After the final extraction, 100 μL of the aqueous phase was used for scintillation counting. Assays were run in duplicate. All data represent the average of the results from three experiments.

Quantitative real-time PCR: Total RNA was isolated by the acid-guanidinium thiocyanate-phenol method with TRI reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. The total RNA obtained was measured at 260 nm with a spectrophotometer. To synthesize cDNA, 2 μg of RNA and 1 unit of Superscript II (Invitrogen) reverse transcriptase were used, according to the manufacturer's instructions. Real-time PCR was performed using a SYBR green PCR kit, according to the manufacturer's instructions. Primers were designed with Primer Express as follows: ACTBF321: 5'-AGAAAATCTGGCACCACACC-3'; ACTBR462: 5'-GGGGTGTTGAAGGTCTCAAA-3', GI L5016088; hSULT2A1F163: 5'-TGAGTTCGTGATAAGGG ATGAA-3'; hSULT2A1R294: 5'-CAGATGGGCACAG AT TGGAT-3', GI L29540544; hSULT1A1F317: 5'-GTGTCACCGAGCTCCCATCT-3', hSULT1A1R395: 5'AGTCTCCATCCCTGAGGGAATC-3', GI L32189358. Real-time PCR was performed on an ABI 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Initially, the regular PCR product DNA was purified with GENECLEAN Turbo (Qbiogene, Carlsbad, CA) for constructing standard curves (10^3-10^8 copies gene). A standard curve was plotted: threshold cycle (C_T) vs. logarithmic value of the gene copy number. The gene copy number of unknown samples was generated directly from the standard curve by Sequence Detector 1.7 software (Applied Biosystems). At least two duplications were run for each experiment and each experiment was repeated at least three times. All gene mRNA copy numbers were normalized to β-actin mRNA values.

Statistical data analysis: In experiments in which SULTs protein/enzyme activity levels in control cells were arbitrarily set to 100%. One-way ANOVA followed by the Dunnett's test was used to calculate the statistical significance of the difference between the control means and DETA/NO treatment means. In all cases, *, $p<0.05$ was considered significant; **, $p<0.01$ was considered very significant. Data presented in the figures are Mean±SD (standard deviation) of the data collected separately from at least three individual animals.

RESULTS

Time-dependent effect of DETA/NO on human SULT2A1 and SULT1A1 protein expression: Human hepatocarcinoma (Hep G2) cells were treated with 1 mM DETA/NO for 2, 3 and 5 days. The medium was changed every 24 h with or without (control) DETA/NO. As shown in Fig. 1, this treatment significantly downregulated SULT2A1 protein expression in a time-dependent manner. Human SULT1A1 protein expression, however, was not significantly affected by this treatment. [^{3}H]DHEA (diluted to 0.4 Ci/mmol; final concentration 2 μM) was used to assay the effect of DETA/NO on DHEA sulfation activity. DETA/NO treatment significantly decreased DHEA sulfation activity in Hep G2 cells (71, 60 and 63%, respectively for 2, 3 and 5 day treatment).

SULT2A1 has high enzyme activity for DHEA; however, there are other SULT isoforms that also show activity toward DHEA. The enzyme activity assay results did not completely agree with the Western blot results for SULT2A1. There is not a substrate that is specific only for SULT2A1.

Concentration-dependent effect of DETA/NO on human SULT2A1 protein and mRNA expression: DETA/NO significantly downregulated both protein and mRNA expression of human SULT2A1 in Hep G2 cells in a concentration-dependent manner at media concentrations of 1 μM to 1 mM (Fig. 2a, b). DETA/NO treatment, however, did not significantly affect human SULT1A1 expression (data not shown). The downregulation of human SULT2A1 protein levels was in very good agreement with that of mRNA levels. The results suggest that DETA/NO downregulation of SULT2A1 occurs at the transcriptional level.

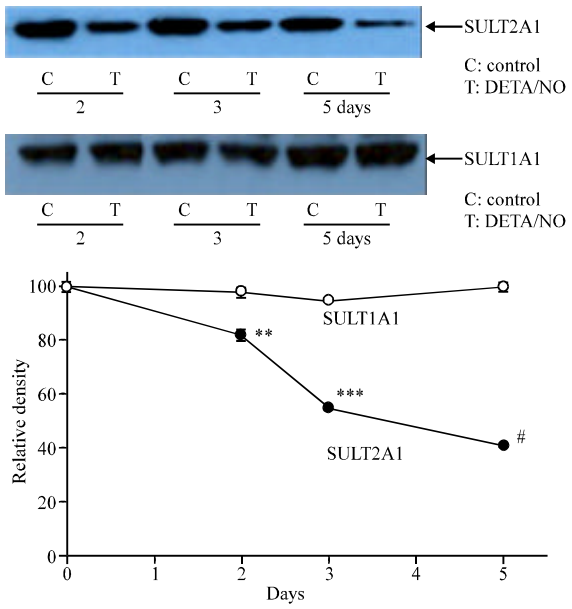

Fig. 1: Time-dependent effect of nitric oxide on SULT2A1 and SULT1A1 protein expression in Hep G2 cells. Hep G2 cells were treated with 1 mM DETA/NO for the number of days indicated. Ten micrograms of protein were used for Western blotting. The densitometric value of the control protein band was arbitrarily set to 100 and densitometric values of DETA/NO-treated samples were calculated relative to the control. Data are Means±SD of three independent experiments. **p<0.01, ***p<0.001, #p<0.0001 when compared with the control

ERK involvement in the DETA/NO downregulation of human SULT2A1: Mitogen-activated protein kinases (MAPKs) are important components of signaling pathways involved in translating extracellular stimuli into a wide range of cellular responses. Therefore, we investigated ERK for its possible involvement in the DETA/NO regulation of human SULT2A1. ERK was significantly activated by 1 mM DETA/NO treatment in a time-dependent manner (Fig. 3). ERK activation was very effectively inhibited by the ERK-specific inhibitor PD98059 (10 μM) (Fig. 4) in the presence (lane D) or absence (lane C) of 1 mM DETA/NO. Most importantly, the inhibition of the DETA/NO-mediated activation of ERK also recovered SULT2A1 protein expression (Fig. 4; compare lanes B and D). These results suggested that MAPK signaling pathways (specifically, ERK) are involved in the DETA/NO downregulation of human SULT2A1.

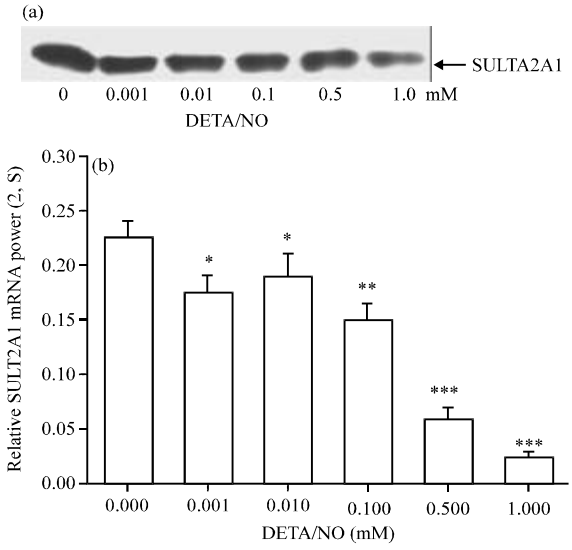

Fig. 2: Concentration-dependent effect of nitric oxide on SULT2A1 protein and mRNA expression in Hep G2 cells. Human Hep G2 cells were treated with different concentrations of DETA/NO as indicated for 1 day. (a) Western blots. Ten micrograms of protein were used for each lane. (b) Relative mRNA expression obtained by q-RT-PCR. To synthesize cDNA, 2 μg of RNA was used and RT-PCR was performed using a gene-specific primer. Human β-actin was used as a control. Data are Mean±SD of 3 independent experiments. **p<0.01 DETA/NO-treated cells compared to control; ***p<0.001 when compared to control

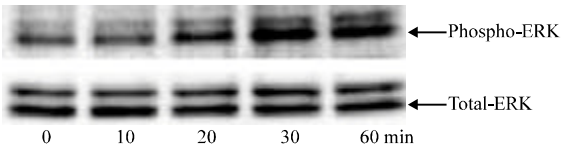

Fig. 3: Time-dependent effect of DETA/NO on the phosphorylation of ERK. Hep G2 cells were treated with 1 mM DETA/NO and allowed to grow for different lengths of time as indicated and then total cell lysates were analyzed for phospho-ERK and total-ERK by Western blotting. Fifteen micrograms of the protein were loaded into each lane. Representative results are shown. Similar results were obtained in two other independent experiments

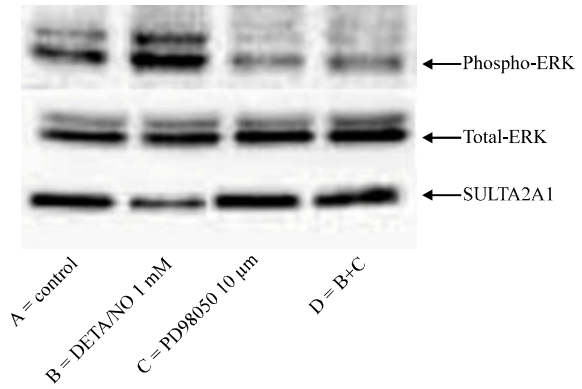

Fig. 4: Effect of PD98059 on the phosphorylation of ERK and on the DETA/NO downregulation of SULT2A1. (a) Hep G2 cells were treated with solvent for 2 days, (b) 1 mM DETA/NO for 2 days; © 10 μM PD98059 for 2 days and (d) or 10 μM PD98059 for 1 h and then 1 mM DETA/NO for 2 days (D). Fifteen micrograms of the protein were used for Western blotting. Representative results are shown. Similar results were obtained in two other independent experiments

DISCUSSION

SULTs have been recognized as important phase II drug-metabolizing enzymes. However, the true biological functions of SULTs are not well understood. From the chemist's point of view, sulfation is one of the most efficient ways to alter the chemical properties of molecules. It is reasonable to believe that sulfation may be one of the important pathways for regulating the biological activities of biologically active molecules. SULTs should play important roles in cellular process and in maintaining the health status of human beings. Dysregulation of human SULTs by xenobiotic drugs, food components and pathological conditions could lead to malfunction of biosignaling molecules, leading to disease. In the present study, we used nitrosative stress (DETA/NO) to disrupt a model biological system (i.e., Hep G2 cells) and to determine the effects of stress on the expression of SULT1A1 and SULT2A1, the two major SULT isoforms. Our results add a new dimension to potential physiological and pathological modulation of SULT2A1.

NO free radical has pleiotropic functions in pathophysiology (Zaki *et al*., 2005). Endogenously synthesized NO can be helpful for health, defending the body through its antimicrobial and cytoprotective activities. In contrast, NO-mediated inflammation and pathogenesis is also known. A predominant view is that excessive NO exerts cytotoxic effects in various cell types by reacting with superoxide, thereby generating the highly reactive free radical peroxynitrite. This causes nonspecific DNA, protein and lipid damage (Brune *et al*., 1999). NO-generated nitrosative stress is also known to activate MAPKs (Obara and Nakahata, 2010; Yndestad *et al*., 2010). MAPKs are important signaling proteins that translate extracellular stimuli into a wide range of cellular responses (Wagner and Nebreda, 2009). MAPK signaling pathways are triggered by many extracellular stimuli, such as growth factors, cytokines and various environmental stresses.

Our experimental results demonstrated that nitrosative stress (DETA/NO) strongly downregulates human SULT2A1 but does not significantly affect human SULT1A1. SULT2A1 catalyzes the sulfation of a wide range of alcohol molecules. However, it has high specific activities toward various hydroxysteroids. Therefore, the downregulation of SULT2A1 may cause the malfunction of various hydroxysteroids. SULT1A1 is a phenolic-specific SULT that has very broad and high activity toward xenobiotic phenols. SULT1A1 is a major enzyme for xenobiotic detoxification. Nitrosative stress does not affect SULT1A1 expression.

Our results also demonstrate that the NO downregulation of SULT2A1 involves MAPK (ERK) signaling pathways. DETA/NO activated ERK cell signaling pathways in a time-dependent manner. Our results showed that the effect of NO on SULT2A1 protein expression was reversed by PD98059, a specific inhibitor of ERK activation. This indicates that NO regulation of hSULT2A1 involves ERK cell signaling pathways. P38 MAPK involvement in phenolic acid induction of human SULT1A1 has been reported (Yeh and Yen, 2006). MAPKs are known to be upregulated in various human

tumors (Wagner and Nebreda, 2009). The exact mechanism for how ERK activation is involved in SULT2A1 gene suppression is not known and need further investigation. Our data suggested that ERK activation by DETA/NO treatments occurs in hours, while SULT2A1 gene suppression by DETA/NO treatments occurs in days. Other cellular processes which are regulated by ERK activation may be involved in the suppression of SULT2A1 expression.

NO of both endogenous and exogenous origin had been reported to down-regulate the rat hydroxysteroid SULT2B1a (pregnenolone specific SULT) using the rat C6 glioma cell line (Kohjitani *et al*., 2008). This cell line does not express SULT2A1 (DHEA specific SULT) or SULT2B1b (cholesterol specific SULT). It was determined that activation of the AMPA receptors inhibits expression of rat SULT2B1a by a process involving intracellular NO signaling (Kohjitani *et al*., 2008). To the best of our knowledge, no other results on NO regulation of SULTs have been reported. Our results, for NO down regulation of human hydroxysteroid SULT2A1 in human Hep G2 cell line agree with the reported results for NO regulation of rat hydroxysteroid SULT2B1a. Hydroxysteroids are sulfated *in vivo* by SULT isoforms. Hydroxysteroids and their sulfates play an important role in nervous and endocrine systems. The specific down regulation of hydroxysteroid SULTs by NO signaling, but not the phenol specific SULT1A1, suggest the potential roles for NO signaling in the regulation of hydroxysteroid functions. This is an interesting fact. Although our results suggest that MAPK signaling pathways may involve in the NO regulation of human SULT2A1 in Hep G2 cells, further mechanism studies for this fact will be significant for understanding the biological functions of SULTs and the regulation of hydroxysteroid functions. SULTs play important roles in hormone regulation, drug metabolism, xenobiotic detoxification, cancer prevention and causation.

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