Extracelllar Oxidation by Taurine Chloramine Activates ERK via the Epidermal Growth Factor Receptor*

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Taurine is present in high concentrations in neutrophils, and when the cells are stimulated taurine can react with hypochlorous acid (HOCl) to form taurine-chloramine (Tau-Cl). This compound retains oxidant activity and can affect the neutrophil itself or surrounding tissue cells. We have investigated the effects of Tau-Cl on MAPK signaling in human umbilical vein endothelial cells (HUVEC). Tau-Cl caused no loss in intracellular glutathione or inactivation of the thiol-sensitive enzyme glyceraldehyde-3-phosphate dehydrogenase, indicating that it had not entered the cells. However, stimulation of HUVEC with Tau-Cl (20–100 μM) induced the rapid activation of ERK within 10 min. This activation was abolished by inhibition of MEK by U0126, indicating that it was not because of direct oxidation of ERK. No activation of p38 was detected. These results suggest that Tau-Cl reacts with a cell membrane target that results in intracellular ERK activation. Tau-Cl over the same concentration range and time scale stimulated epidermal growth factor (EGF) receptor tyrosine phosphorylation in A431 cells and HUVEC. The EGF receptor inhibitor PD158780 significantly attenuated Tau-Cl-induced phosphorylation of both the EGF receptor and ERK. This implicates the EGF receptor in the upstream activation of ERK. The Src tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(3-butylo)pyrazolol[3,4-d]pyrimidine had no effect on Tau-Cl-induced EGF receptor or ERK activation. We propose that Tau-Cl acts on an oxidant-sensitive target on the cell surface, this being either the EGF receptor itself or another target that can interact with the EGF receptor, with consequential activation of ERK.

Cells respond to oxidative stress through the activation of multiple signaling pathways that control responses ranging from proliferation and transformation to growth arrest, apoptosis, or cell death (1–3). Critical signaling pathways involved in all these processes are the phosphorylation cascades leading to the activation of mitogen-activated protein kinases (MAPK),1 including extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (4–6). Oxidative stress also activates tyrosine kinases (2). The epidermal growth factor (EGF) receptor is one such tyrosine kinase that is activated by various oxidants (7, 8).

Although it is well recognized that reactive oxidants such as hydrogen peroxide, hypochlorous acid, superoxide, nitric oxide, and peroxynitrite can activate cell-signaling pathways, the mechanisms by which this occurs is not fully understood. One likely mechanism involves modification of reduced thiol proteins either through oxidation or nitrosylation. Oxidation may be direct or a consequence of changes in the redox state within the cells and may be regulated through antioxidant pathways involving glutathione or thioredoxin (9). However, despite intensive investigation of oxidant-sensitive processes, the critical targets have not been identified.

Neutrophils are a major source of biological oxidants. When stimulated, these cells produce vast amounts of superoxide that dissipates to H2O2. The enzyme myeloperoxidase utilizes H2O2 and chloride to produce hypochlorous acid (HOCl) (10), a potent oxidant that reacts with a wide range of biological targets (11, 12), and is implicated as a cause of inflammatory tissue damage (13). Thiols and thioethers are most readily oxidized by HOCl, and this oxidant also reacts with amino groups to generate chloramines (Reaction 1).

\[
\text{HOCl} + \text{RNH}_2 \rightarrow \text{RNHCl} + \text{H}_2\text{O}
\]

**Reaction 1**

Chloramines retain many of the oxidizing properties of HOCl, showing a high reactivity with thiol groups, and can oxidize thiethers and heme proteins (14–16). The cytotoxicity of these oxidants depends on their structure and their ability to cross the plasma membrane (17, 18). HOCl and small uncharged chloramines like monochloramine (NH2Cl) are readily permeable across cell membranes and are cytotoxic at low concentrations (15). Other chloramines, however, are much less permeable, and their toxicity varies accordingly. Glycine chloramine and histamine chloramine enter cells slowly and react with intracellular constituents (19, 20), whereas taurine chloramine (Tau-Cl; \(\text{SO}_2\text{CH}_2\text{CH}_2\text{NHCl}\)), which is charged and much less membrane-permeable, has low toxicity toward a number of cell types (15, 21, 22).

The formation of Tau-Cl and its low permeability are of interest for several reasons. First, it provides a model of a chlorinated oxidant that will be restricted to the outside of the cell, and therefore allows investigation of oxidant-sensitive membrane targets. Second, neutrophils contain high concentrations of taurine (23), some of which is released and reacts with hypochlorous acid to form taurine-chloramine.

\[
\text{REACTION 2}
\]

**Reaction 2**

\[\text{HOCl} + \text{H}_2\text{O} \rightarrow \text{HO}_2\text{Cl} \rightarrow \text{HCl} + \text{H}_2\text{O}_2\]

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Tau-Cl-induced Signaling via EGF Receptor

becomes chlorinated when the cells are stimulated (15, 22, 24). Therefore, it is likely that tauine is one of the physiological targets for chlorination by HOCl, leading to the generation of Tau-Cl at inflammatory sites. It has been proposed that Tau-Cl formation is a detoxification mechanism for HOCl, providing protection against neutrophil-induced cytotoxicity (25). However, Tau-Cl is also a reactive oxidant, and more recent reports suggest that it can affect cell signaling pathways. It has been shown to inhibit activation of NFκB and production of NO, tumor necrosis factor-α, and other pro-inflammatory molecules in various cell models (26–28) and to induce cell growth arrest and activation of p53 (29). How Tau-Cl mediates cell signaling has not been determined.

We and others have shown that HOCl and some chloramines are capable of causing proliferation, cell cycle arrest, and apoptosis in a wide range of cells (29–32) and that HOCl at sub-lethal concentrations is capable of activating ERK and p38 (33). Given that many of the physiological effects of HOCl are likely to occur via the intermediacy of chloramines, we have investigated the ability of Tau-Cl to activate MAPK pathways in endothelial cells. We demonstrate that Tau-Cl activates ERK but not p38, and we provide evidence for the involvement of the EGF receptor in this process.

MATERIALS AND METHODS

Reagents—Cell culture media and supplies were from Invitrogen. Primary antibodies directed against anti-phospho-ERK1/2, phospho-p38, and phosphate-Jun N-terminal kinase were purchased from New England Biolabs, Inc. (Beverly, MA). The anti-phosphotyrosine (PY20) antibody was from Zymed Laboratories Inc. (San Francisco, CA), and anti-ERK and anti-EGF receptors were from Sigma. The MAPK/ERK kinase (MEK)-specific inhibitor U0126, EGF receptor-specific inhibitor PD188380, and the Src family-specific inhibitor 4-amino-5(4-chlorophenyl)-7-(butyl)pyrazolo[3,4-d]pyrimidine (PP2) were obtained from Calbiochem. Complete™ protease inhibitors were from Roche Applied Science. Reagent HOCl was supplied by Sara Lee (Auckland, New Zealand). All other chemicals unless stated were purchased from Sigma.

Preparation of Tau-Cl—Tau-Cl was freshly prepared on the day of use by adding 2 mM NaOCl dropwise to 10 mM tauine in PBS, with vigorous vortexing. Chloramine was monitored by UV absorption spectra (200–400 nm) to ensure the absence of dichloramine and unreacted HOCl (34). Tau-Cl concentration was determined spectrophotometrically using ε253 nm = 415 mM–1 cm–1. Alternatively, the concentration of HOCl and chloramines was determined by reaction with 5-thio-2-nitrobenzoic acid, and the change in absorbance was measured at A412 (ε = 28,200 M–1 cm–1). The 5-fold excess of tauine ensured that only the monochloramine and no dichloramine was formed.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained with informed consent, harvested, and grown in M-199 media supplemented with 15% fetal calf serum and growth supplements as described previously (35). Cells were used in the second or third passage after primary culture. A431 cells that have a greater EGF receptor density were utilized in some experiments. They were purchased from American Type Culture Collections (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

Western Blotting—After treatment, cells were washed three times with ice-cold PBS and lysed with Nonidet P-40 lysis buffer (25 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1.6 mg mL–1 Complete™ protease inhibitors (Roche Applied Science), 1 mM sodium vanadate, and 0.5% Nonidet P-40) for 15 min on ice. Insoluble material was removed by centrifugation at 13,000 × g for 5 min. Equal amounts of proteins from cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were probed with antibodies against dually phosphorylated ERK1/2, p38 (New England Biolabs), phosphotyrosine PY20 (Zymed Laboratories Inc.), EGF receptor, or ERK (Sigma) according to the manufacturer's instructions. A horseradish peroxidase-conjugated secondary antibody was used to visualize the immunoblotted proteins through enhanced chemiluminescence. Protein concentrations were determined by the Bradford (Bio-Rad) assay using bovine serum albumin as a standard.

RESULTS

Low Cytotoxicity and Cell Permeability of Taurine Chloramine—To establish that the low cell permeability of Tau-Cl applies to HUVEC, the rate of oxidant consumption by the cells was measured, and the activities of GAPDH and the intracellular GSH concentration were monitored. GAPDH is a thiol enzyme that is particularly sensitive to oxidants, provided they can penetrate the cells (35). Very little Tau-Cl was consumed from the medium during incubation with HUVEC, with 90% remaining after 1 h (Fig. 1A), whereas HOCl was consumed within 20 min. Little HOCl was consumed when cells were not present. Tau-Cl showed no cytotoxic effects at any of the time points or concentrations investigated, as determined by flow cytometry with propidium iodide (data not shown). Morphologically, HUVEC also appeared unchanged when treated with up to 500 μM of Tau-Cl. There was no membrane shrinkage, and the cells remained firmly attached to the plate.

There was no detectable decrease in GAPDH activity (Fig. 1B) or significant loss of GSH (Fig. 1C) on treating the cells for 10 min with up to 100 μM Tau-Cl. This contrasts with HOCl, which under the same conditions causes 50% inactivation of GAPDH at 18 μM, complete inactivation with 50 μM, and 50% loss of GSH at 40 μM (Fig. 1C) (35). The inability of Tau-Cl to inactivate intracellular GAPDH was not because of a lack of reactivity toward the enzyme. When HUVEC were lysed before the addition of Tau-Cl and then assayed for GAPDH activity, total inhibition of GAPDH activity was achieved with less than 10 μM chloramine (Fig. 1B). These results confirm the low permeability of Tau-Cl and its inaccessibility to intracellular targets. The lack of GSH oxidation also indicates that Tau-Cl does not affect the overall thiol redox state through transmembrane electron transfer.

Tau-Cl Activates ERK—Various members of the MAPK family have been shown to be activated by cell-permeable oxidants such as H2O2 and HOCl (1, 4, 5, 33). To determine whether the ERK and p38 MAPK pathways are activated in HUVEC treated with the impermeable oxidant Tau-Cl, the phosphorylated activated forms of ERK and p38 were analyzed. Activation

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It was significant over the concentration range 20–200 μM Tau-Cl and maximal, on average, at 50 μM (Fig. 2B). Although taurine alone at high concentrations has been shown to activate ERK in osteoblasts (38), we saw no activation of ERK in our system at the concentrations of taurine routinely used in our investigation (Fig. 2A).

To determine whether Tau-Cl acts on ERK directly, cells were pretreated with U0126, a specific inhibitor of MEK, the
kinase directly upstream from ERK, before the addition of Tau-Cl. Activation of ERK by Tau-Cl was fully inhibited by pretreatment with U0126 (Fig. 2D), indicating that Tau-Cl does not act directly on the kinase but must have a target further upstream in this signaling cascade.

**EGF Receptor Phosphorylation by Tau-Cl**—As Tau-Cl was not able to activate ERK directly and was incapable of entering the cell, a potential membrane target of Tau-Cl action was sought. One of the upstream signaling events for the activation of ERK involves the activation of the EGF receptor. To investigate the ability of Tau-Cl to activate the EGF receptor, the EGF receptor overexpressing A431 cell line was used. Activation of the receptor involves the tyrosine phosphorylation at multiple sites depending on the stimulus (39, 40). Treatment of A431 cells with Tau-Cl caused an increase in the tyrosine phosphorylation of the EGF receptor (Fig. 4A) that was evident at 20 μM and maximal at 50 μM oxidant (Fig. 4B). With these Tau-Cl concentrations, no changes were observed in the levels of the immunoprecipitated receptor (Fig. 4A). In a time course experiment, phosphorylation of the EGF receptor occurred within 5 min of Tau-Cl addition, was maximal at 15 min, and maintained for over an hour (data not shown).

Others have shown (41, 42) that activated ERK is capable of phosphorylating the EGF receptor. To determine whether ERK participated in Tau-Cl-induced phosphorylation of the EGF receptor, A431 cells were pretreated with the MEK inhibitor U0126. ERK activation was prevented, but only a minor decrease in the phosphorylation of EGF receptor was seen, suggesting that ERK was not responsible for this effect (data not shown). To establish whether phosphorylation of EGF receptor by Tau-Cl preceded that of ERK, a specific EGF receptor tyrosine kinase inhibitor PD158780 was used. Treatment of A431 cells with this inhibitor dramatically decreased the phosphorylation of EGF receptor by EGF as well as decreasing base-line phosphorylation (Fig. 5A). Phosphorylation of the EGF receptor induced by Tau-Cl was also suppressed by PD158780 (Fig. 5A). Western blotting with an antibody against the EGF receptor confirmed that the receptor band was present, so low recovery cannot explain the low phosphotyrosine reactivity. ERK phosphorylation induced by either EGF or Tau-Cl was completely abolished when EGF receptor activation was inhibited by PD158780 (Fig. 5B). Thus, Tau-Cl-induced activation of ERK appears to require prior activation of the EGF receptor.

The possibility that EGF receptor activation occurred via the Src family kinases (6, 43) was explored by using PP2, a specific Src family kinase inhibitor. PP2 did not inhibit ERK activation by Tau-Cl (Fig. 5C), implying that the activation of ERK by Tau-Cl is dependent on the action of the EGF receptor but is independent of Src family kinase activation.

Similar results were seen when HUVEC were used, but due to the lower EGF receptor numbers the results were less pronounced. As shown in Fig. 5D, Tau-Cl treatment caused EGF receptor phosphorylation that was inhibited by PD158780, and PD158780 also inhibited ERK phosphorylation.
DISCUSSION

In this study, we have shown that the cell-impermeable oxidant Tau-Cl can activate an intracellular signaling pathway. Tau-Cl activated ERK quickly and at low concentrations and also caused tyrosine phosphorylation of the EGF receptor in two cell types. Tau-Cl has been shown by others (15, 21, 22) to have low membrane permeability, and we have provided good evidence that it does not enter HUVEC. It did not alter intracellular GSH levels or GAPDH activity. The only Tau-Cl consumption seen was a small but consistent loss of about 2 μM immediately on adding to the cells, and the continued presence of the oxidant provided no obvious further stress to the cells. We propose that the initial small loss of Tau-Cl was because of reaction with extracellular targets, and oxidation of one or more of these was responsible for activating the signal responses.

ERK phosphorylation was inhibited by U0126, an inhibitor of upstream MEK, indicating that a direct reaction between Tau-Cl and ERK was not responsible for activation. This is consistent with findings that ERK activation by other oxidants is also indirect (7, 44). MEK inhibition did not prevent phosphorylation of the EGF receptor, which suggests that ERK was not responsible for this reaction, as has been observed with NO-treated cells (42). However, the specific inhibitor PD158780, which blocks phosphorylation of the EGF receptor, also prevented ERK activation. Activation of ERK and the EGF receptor occurred over the same time frame and concentration range of Tau-Cl. Taken together, these findings provide strong support for activation of the EGF receptor being the upstream signal for ERK activation (Fig. 6). Our evidence that Tau-Cl did not enter the cells implies that EGF receptor activation occurs by oxidation of an extracellular target. As shown in Fig. 6, this could either be a direct reaction with an accessible site on the receptor itself or a reaction with an alternative membrane target that enables receptor activation.

Several oxidants including H₂O₂ and peroxynitrite induce the phosphorylation of the EGF receptor, albeit at higher concentrations than Tau-Cl (5, 7, 8, 43). In these cases, the oxidants do not appear to act directly on the receptor, but there is no evidence to suggest that they act extracellularly. Activation by H₂O₂ or peroxynitrite is inhibited, at least partially, by Src inhibitors (5, 43). However, this was not the case with Tau-Cl, implying a different mechanism. Most interesting, activation by H₂O₂ results only in tyrosine phosphorylation and not the combined tyrosine, serine, and threonine phosphorylation that occurs with EGF (8). If this were also the case with Tau-Cl, it raises the possibility that a surface tyrosine phosphatase may be inactivated by oxidation.

Although it is well established that various oxidants activate
Receptor activation results in the activation of the receptor and ERK, leading to the activation of signaling pathways involving EGF signaling pathways inducing the phosphorylation of several enzymes as well as transcription factors. MAPK and other cell signaling pathways (1, 7, 45), specific mechanisms and targets for oxidation are not well understood. One proposal is that depletion of cellular GSH and alterations in cell redox potential affect the thiol oxidation status of signaling proteins (9). However, the lack of GSH oxidation makes this mechanism unlikely with Tau-Cl. Alternatively, there is evidence in some cases that inhibition of tyrosine phosphatases such as PTP1B, which contain oxidant-sensitive critical thiol groups, leads to increased phosphorylation of various proteins, including the EGF receptor (6, 45) and consequent signal transduction. However, this is also difficult to rationalize with Tau-Cl activation as phosphatases tend to be located and act intracellularly.

Chloramines are relatively selective oxidants for thiol groups and methionines (16), forming disulfides and sulfides, respectively. Surface-accessible residues of these amino acids would be likely targets for Tau-Cl. The EGF receptor does contain external cysteine-rich regions, but these are considered to contain structural disulfide bonds (46). There are six internal thiols in the kinase domain that have been shown to be targets for thiol reagents such as N-ethylmaleimide, which block kinase activity (47). A role for oxidation of EGF receptor cysteine residues in activation by Tau-Cl is therefore not readily apparent. Further investigation is needed to determine specifically whether the EGF receptor or other proteins contain cysteines or methionines at or near the cell surface whose oxidation is associated with receptor activation.

A number of studies using human leukocytes, fibroblasts, and synoviocytes have shown that pretreatment with Tau-Cl decreases the ability of the cells to up-regulate production of inflammatory cytokines and messengers such as IL-8, IL-10, tumor necrosis factor, and nitric oxide (26, 27, 48–50). This may involve inhibition of activation of the transcription factor, NFκB (49, 51). The findings of Kanayama et al. (28) that high concentrations of Tau-Cl oxidized a methionine residue in its inhibitor, IκB, and prevented IκB degradation support this mechanism. At first, this action appears contrary to the evidence that Tau-Cl does not enter cells. However, the experimental conditions used in these studies differ markedly from ours. Their Tau-Cl concentrations were about 10-fold higher; incubation times were much longer, and most important, the studies were performed in full medium. As the medium is rich in amino acids, chlorine exchange could occur to give more permeable species that are capable of reaching intracellular targets.

Our results have implications for the physiological role of neutrophil-derived oxidants. Neutrophils contain about 20 mM taurine, some of which is released and able to trap HOCl when the cells are stimulated (22). At inflammatory sites, taurine and amino groups on surrounding proteins are likely to be targets for the HOCl produced by neutrophils. Like Tau-Cl, protein-bound chloramines should be membrane-impermeable. Our findings suggest that rather than being protective, formation of these chloramines could contribute to the cellular responses to inflammation by activating the EGF receptor and the ERK arm of the MAPK pathway. In particular, they could contribute to the endothelial dysfunction seen at sites of chronic inflammation such as atherosclerotic plaques (52).

In summary, we have shown that Tau-Cl activates ERK and that this is most likely a consequence of EGF receptor activation via oxidation of a cell surface target. The pattern of MAPK activation is different for Tau-Cl compared with HOCl and other oxidants, reinforcing the point that reactive oxygen species do not all have the same cellular effects. As individual chloramines differ in their reactivity and cell permeability, they may also elicit different cellular responses.

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