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Original Contribution

Oxidative stress induces actin-cytoskeletal and tight-junctional alterations in hepatocytes by a Ca^{2+}-dependent, PKC-mediated mechanism: Protective effect of PKA

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Abstract

Oxidative stress elevates Ca^{2+} and, presumably, activates Ca^{2+}-dependent PKCs. We analyzed the participation of Ca^{2+}-dependent PKCs in actin disorganization and tight-junctional impairment induced by the pro-oxidant *tert*-butylhydroperoxide (*t*BOOH) in isolated rat hepatocyte couplets. *t*BOOH (100 μM) augmented radical oxygen species (ROS), as indicated by increased lipid peroxidation (+217%, $p < 0.05$) and intracellular production of 2′,7′-dichlorofluorescein (+36%, $p < 0.05$). Cytosolic Ca^{2+} and PKCα translocation to membrane, an indicator of PKCα activation, were also elevated by *t*BOOH (+100 and +79%, respectively, $p < 0.05$). *t*BOOH increased the number of couplets displaying membrane blebs (+278%, $p < 0.001$) and caused redistribution of F-actin. *t*BOOH induced tight-junctional impairment, as indicated by a reduction in the percentage of couplets retaining presecreted cholyllysylfluorescein in their canalicular vacuoles (−54%, $p < 0.001$). *t*BOOH induced redistribution of the tight-junctional-associated protein ZO-1. All these events were prevented by the panspecific PKC inhibitors H7 and staurosporine, the Ca^{2+}-dependent PKC inhibitor Gö6976, the intracellular Ca^{2+} chelator BAPTA/AM, and the PKA activator dibutyryl-cyclic AMP. Furthermore, PKC inhibition and PKA activation not only prevented but also fully reversed *t*BOOH-induced blebbing. Conversely, *t*BOOH-induced ROS formation and Ca^{2+} elevation remained unchanged. We conclude that ROS induce hepatocellular actin-cytoskeleton rearrangement and tight-junctional impairment by a PKC-mediated, Ca^{2+}-dependent mechanism, which is counteracted by PKA.
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Oxidative stress (OS) induces cellular injury by the production of free radicals of high reactivity, leading to membrane lipid peroxidation [1] and oxidation of critical thiol groups, causing formation of homo- and heteroproteins bound by disulfide bridges and inhibition of their functions [2].

In recent years, evidence has been accumulated indicating that OS is involved in hepatopathies, such as ischemic liver [3], alcoholism [3], steatohepatitis [4], and pathologies leading to hepatic iron accumulation [5], among others. OS induces differential hepatocellular alterations, depending on its intensity and duration. Levels low enough to maintain ATP levels cause early alterations of hepatocanalicular function [6,7] and,

Abbreviations: OS, oxidative stress; IRHC, isolated rat hepatocyte couplet; *t*BOOH, *tert*-butylhydroperoxide; Men, menadione; CLF, cholyllysylfluorescein; PKC, protein kinase C; L-15, Leibovitz-15; DB-cAMP, N^6,2′-*o*-dibutyryladenosine 3′:5′-cyclic monophosphate; SP, staurosporine; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; Fura-2/AM, fura-2 pentakis(acetomethyl) ester; LDH, lactate dehydrogenase; ROS, radical oxygen species; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; GSHt, total glutathione; GSSG, oxidized glutathione; $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} concentration; cVR, canalicular vacuolar retention; PKA, protein kinase A; MAPKs, mitogen-activated protein kinase.

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eventually, apoptosis [8]. Higher levels lead to dramatic changes in plasma membrane permeability, release of cytosolic and mitochondrial components, impaired mitochondrial ATP production, and finally, necrosis [8].

We have characterized a number of early hepatobiliary dysfunctions induced by low, prenecrotic levels of OS, relevant to bile formation. Using isolated rat hepatocyte couplets (IRHCs), an in vitro model for the study of polarized plasma bile transport, we have shown that two different OS inducers, the synthetic hydroperoxide *tert*-butylhydroperoxide (*t*BOOH) [6] and the redox-cycling quinone menadione (Men) [7], induce a dose- and Ca^{2+}-dependent decrease in the IRHC capability to secrete and retain the fluorescent bile salt analogue, cholyllysylfluorescein (CLF), in their canalicular vacuoles. These alterations were accompanied by membrane bleb formation, disarray of the F-actin cytoskeleton, and impairment of tight-junctional permeability [6,7].

Because OS-induced injury occurs with elevations of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) [7,9], and the so-called "classical" protein kinases C (PKCs) require Ca^{+2} for activation [10], our studies raise the possibility that PKC activation mediates OS-mediated cellular injury. If so, PKC-counteracting agents should prevent hepatocellular alterations induced by OS; examination of this hypothesis was the main aim of the present study.

Material and methods

Materials

CLF was kindly provided by Dr. Charles O. Mills (Birmingham, UK). Collagenase type A from *Clostridium histolyticum* was purchased from Gibco (Paisley, UK). Leibovitz-15 (L-15) tissue culture medium, BSA (fraction V), *t*BOOH, N^6,2′-*o*-dibutyryladenosine 3′:5′-cyclic monophosphate (DB-cAMP), H7, staurosporine (SP), dimethyl sulfoxide (DMSO), and fluorescein isothiocyanate (FITC)-labeled phalloidin were from Sigma Chemical Co. (Poole, Dorset, UK). KT5720, BAPTA/AM, and Gö6976 were from Allexis Co. (Bingham, Nottingham, UK). Rabbit anti-ZO-1 and FITC-labeled goat anti-rabbit IgG were from Zymed (San Francisco, CA, USA). *N,N*′-diphenyl-*p*-phenylendiamine and fura-2 pentakis(acetomethyl) ester (Fura-2/AM) were obtained from Fluka Chemika (Steihem, Switzerland). 2′,7′-dichlorofluorescin diacetate was from Molecular Probes (Eugene, OR, USA). All other chemicals were of reagent grade.

Animals

Male Wistar rats (210–250 g) were used throughout. Animals had free access to a standard maintenance diet (41B; Pilsbury, Birmingham, UK) and tap water. Rats were anesthetized using ketamine hydrochloride (Ketalar) 6 mg/100 g body wt, with medetomidine (Domitor) 25 μg/100 g body wt. All animals received humane care according to the criteria outlined in the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health Publication 25-28, revised 1996). The experimental protocols were approved by the Ethics Committees of both the University of Birmingham and the University of Rosario.

IRHC isolation, enrichment, and culture

Hepatocyte IRHCs were obtained according to a two-step collagenase perfusion procedure [11]. This initial preparation was further enriched by centrifugal elutriation [12]. The resulting preparation, containing 67 ± 5% IRHCs (viability >95%), was plated in L-15 containing 50 U/ml penicillin and 50 μg/ml streptomycin onto 35-mm plastic culture dishes (2 ml/dish), at a density of 5×10^4 units/ml, and incubated at 37°C for 4.5 h.

Evaluation of tBOOH effect on hepatocellular integrity

Hepatocyte viability was assessed by the trypan blue exclusion test [13].

Plasma membrane integrity was evaluated by the leakage of the cytosolic enzyme lactate dehydrogenase (LDH; EC 1.1.1.27). LDH activity was assessed spectrophotometrically (Perkin–Elmer UV/Vis Spectrometer Lambda2S; Überlingen, Germany) by measuring NADH consumption at 340 nm, using a commercial kit (Wiener Laboratories, Rosario, Argentina).

ATP content was measured by the luciferin–luciferase method [14], using a luminescence spectrometer (Perkin–Elmer LS-50B; Buckinghamshire, UK).

Evaluation of tBOOH-induced OS

The magnitude of OS induced by *t*BOOH was evaluated both directly by assessing fluorimetrically the intracellular production of radical oxygen species (ROS) or, indirectly, by measuring generation of lipid peroxidation products.

ROS formation was assessed by using 2′,7′-dichlorofluorescein as a fluorophore; its nonfluorescent, parent compound, 2′,7′-dichlorofluorescin diacetate, readily crosses the hepatocyte membrane and is metabolized by nonspecific esterases and oxidized to 2′,7′-dichlorofluorescein by intracellular ROS [15]. 2′,7′-Dichlorofluorescein was detected fluorimetrically at 488/525 nm excitation/emission wavelengths, using a Shimadzu RF-5301 PC spectrofluorophotometer (Shimadzu Corp., Kyoto, Japan). For this purpose, 3 ml of a hepatocyte suspension containing 3.3×10^3 cells/ml was preloaded with 20 μM 2′,7′-dichlorofluorescin diacetate for 30 min, washed twice, and resuspended in the incubation medium containing *t*BOOH (100 μM) for 15 min before fluorimetric detection.

Lipid peroxidation was evaluated by a modification of the thiobarbituric acid-reactive substances (TBARS) method [16], with minor modifications [17]. A standard curve using 1,1,3,3-tetramethoxypropane, which is converted mole for mole into malondialdehyde (MDA), was routinely run. Protein content in the aliquots of cell suspension used for the assay was measured by the method of Lowry et al. [18]. TBARS were then expressed as nanomoles of MDA equivalents per milligram of proteins.

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