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Indomethacin induces endoplasmic reticulum stress, but not apoptosis, in the rat kidney

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in clinical practice. However, their use is often associated with adverse effects in the gastrointestinal tract and kidney. Our earlier work with indomethacin, a prototype NSAID, has shown that it induced oxidative stress in the kidney in rats, an event that has been postulated to contribute to pathogenesis of its adverse effects in this organ. Endoplasmic reticulum (ER) stress responses have been shown to occur in response to oxidative stress. We investigated whether this occurred in the rat kidney, in response to indomethacin. For this, Wistar rats were orally gavaged with indomethacin (20 mg/kg). Markers of ER stress were studied in the kidneys 1, 12 and 24 h later. GRP78, p-PERK and nuclear sXBP-1, all markers of ER stress, were found to be increased in the rat kidney at 12 h, in response to indomethacin; levels of these markers fell by 24 h. The effects seen at 12 h were attenuated by pre-treatment with zinc, a known anti-oxidant, which has earlier been shown to ameliorate indomethacin-induced oxidative stress. Activation of an ER stress response was not associated with induction of apoptosis, as measured by markers of apoptosis such as release of cytochrome c from mitochondria into the cytosol, activation of caspases 3 and 9, cleavage of poly-ADP ribose polymerase and the presence of DNA laddering. We conclude that indomethacin-induced oxidative stress activated ER stress, but did not lead to apoptosis in the rat kidney.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) often cause adverse effects in the gastrointestinal tract and kidneys (Epstein, 2002; Gabriel et al., 1991). Indomethacin, a prototype NSAID, has been shown to induce oxidative stress and mitochondrial dysfunction in the kidney (Basivireddy et al., 2004; Varghese et al., 2009). Reactive oxygen species (ROS) are known to break protein disulfide bonds, resulting in accumulation of unfolded or misfolded proteins in cells (Inagi, 2009). Accumulation of these unfolded proteins in the endoplasmic reticulum activates a repair mechanism known as endoplasmic reticulum stress (ER stress) response or unfolded protein response (UPR) (Kitamura, 2008; Malhotra and Kaufman, 2007; Yoshida, 2007). The ER stress response broadly consists of two distinct but inter-related pathways: the "light" and "dark" pathways (Inagi, 2009; Kitamura, 2008; Yoshida, 2007). The light pathway involves cellular adaptation to stress by translational suppression, induction of ER chaperones (such as 150 kDa oxygen-regulated protein [ORP150], 78 kDa glucose-regulated protein [GRP78], GRP94, calreticulin) and ER-associated degradation [ERAD] to eliminate immature proteins or unfolded proteins by proteasomal degradation (Vembar and Brodsky, 2008; Yoshida, 2007). These responses collectively attempt to rescue the cell from damage induced by the stress. On the other hand, severe and prolonged stress that is likely to result in cellular damage that is beyond repair results in activation of a second pathway, called the "dark" pathway (Inagi, 2009; Kitamura, 2008; Yoshida, 2007). This results in induction of apoptosis and eventually cell death (Inagi, 2009; Kitamura, 2008; Yoshida, 2007).

Accumulation of unfolded proteins in the ER triggers the release of GRP78 from trans-membrane ER proteins, such as inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK) and activating transcription factor-6 (ATF6) (Inagi, 2009). IRE1 is an endonuclease that catalyzes alternative splicing of mRNA for X-box-binding protein (XBP). This leads to a functionally active transcription factor, sXBP-1, that initiates transcription of ER stress response genes (Hahmann et al., 2011; Inagi, 2009). PERK inhibits protein translation by phosphorylation and resultant inactivation of eukaryotic initiation factor-2α (eIF2α) (Malhotra and Kaufman,

Abbreviations: ATF4, activating transcription factor-4; ATF6, activating transcription factor-6; CHOP, CCAAT/enhancer binding protein-homologous protein; cyt c, cytochrome c; eIF2α, eukaryotic translation initiation factor-2α; ER stress, endoplasmic reticulum stress; GRP 78, glucose-regulated protein 78; HO-1, heme oxygenase-1; IRE1, inositol-requiring enzyme 1; Nrf2, nuclear factor E2 related factor 2; NSAID, non-steroidal anti-inflammatory drug; PARP, poly-ADP ribose polymerase; PERK, double-stranded RNA-activated protein kinase-like ER kinase; ROS, reactive oxygen species; sXBP, spliced form of X-box-binding protein

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2007). Both ATF-6 and ATF4 have been shown to be activated by ER stress and this, in turn, activates apoptosis (Kim et al., 2011; Kitamura, 2008; Carlisle et al., 2014).

Indomethacin has been shown to activate ER stress responses and apoptosis in various cells *in vitro* (Suemasu et al., 2009; Tsutsumi et al., 2004; Okamura et al., 2008; Franceschelli et al., 2011). However, more recently, Matsumoto et al. (2013) have shown that apoptosis did not occur in HepG2 cells, in response to ER stress. It is not known whether ER stress and/or apoptosis occur in the kidney *in vivo* following indomethacin administration.

Zinc, a transition metal that has powerful anti-oxidant effects (Powell, 2000), has been shown to ameliorate indomethacin-induced oxidative stress in the rat kidney (Varghese et al., 2009) and small intestine (Basivireddy et al., 2002; Sivalingam et al., 2011). However, it is not known whether pre-treatment with zinc affects a possible ER stress response, following indomethacin administration.

2. Materials and methods

2.1. Materials

Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), bovine serum albumin (BSA), TRI reagent, ammonium persulphate, N,N,N′,N′-tetramethylethylenediamine (TEMED), 2-mercaptoethanol, sodium dodecyl sulfate (SDS), protease inhibitors (aprotinin, leupeptin and phenylmethylsulfonyl fluoride [PMSF]), phosphatase inhibitors (sodium orthovanadate and sodium fluoride), 7-amino-4-trifluoromethylcoumarin (AFC), caspase-3 substrate (Ac-DEVD-AFC), caspase-9 substrate (Ac-LEHD-AFC) and primary antibody for β-actin were purchased from Sigma, St. Louis, USA. Agarose was purchased from Genei, Bangalore, India. Zinc sulfate was purchased from Sarabhai Merck Limited, Baroda, India. Cell strainer of 100 μM pore size was purchased from BD Biosciences, California, USA. ApoAlert caspase-3 fluorescent assay kits were from Clontech Laboratories, CA. ECL DualVue western blotting markers were purchased from GE Healthcare Bio-sciences Corp, Piscataway, USA. Primary antibodies for GRP78 (cat# SC 1051), XBP-1 (cat# SC 7160), phospho-PERK (cat# SC 32577) and CHOP (cat# SC 575) were purchased from Santa Cruz Biotechnology, Inc., CA, USA. Poly-ADP ribose polymerase (PARP) (cat# 9542) and cytochrome c (cat# 4272) rabbit polyclonal primary antibodies were purchased from Cell Signaling Technology, Inc., Danvers, MA, USA. Rabbit anti-goat IgG (H+L) peroxidase-conjugated (cat# 31402), goat anti-rabbit IgG (H+L) peroxidase-conjugated (cat# 31462), goat anti-mouse IgG (H+L) peroxidase-conjugated (cat# 31430) secondary antibodies and Super Signal West Dura extended duration substrate (cat# 34075) were purchased from Thermo Scientific, IL, USA. Polyvinylidene difluoride (PVDF) membrane (0.45 μm) was obtained from Millipore, Bangalore, India.

2.2. Animals

Male Wistar albino rats (*Rattus norvegicus*) weighing 200–220 g were used for the study. All animals were maintained under conditions of controlled light (12 h light–dark cycles) and temperature (25°C ± 3°C). They had access to standard rodent chow and water *ad libitum*. All experiments performed on the animals were approved by the institutional animal ethics committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Six animals were used in each experimental group.

2.3. Dosing

Rats were administered indomethacin by oral gavage at a dose of 20 mg/kg in 5% sodium bicarbonate. This dose has been standardized in our previous studies and has been shown to consistently produce oxidative stress in the kidney (Basivireddy et al., 2004; Varghese et al., 2009). To study the effects of zinc, zinc sulfate ($ZnSO_4 \cdot 7H_2O$) at a dose of 50 mg/kg body weight (containing 11.4 mg of elemental zinc/kg body weight) (Joseph et al., 1999; Varghese et al., 2009) was administered by oral gavage, 2 h before the dose of indomethacin. Animals were killed by cervical dislocation under halothane anesthesia, at 1, 12 or 24 h after the indomethacin dose. The kidneys were removed, snap-frozen and stored at −70 °C till further use.

2.4. Isolation of cytosolic and nuclear proteins

Cytosolic and nuclear protein extracts were prepared from whole kidney lysates, according to a previously described method (Hershfield et al., 2006). Purity of cytoplasmic and nuclear extracts were assessed by the absence of lactate dehydrogenase (LDH) activity in nuclear extracts and by showing the absence of DNA in cytosolic extracts, as assessed by agarose gel electrophoresis.

2.5. Isolation of rat kidney mitochondria

Preparation of mitochondrial protein extracts was carried out from whole kidney lysates as described previously (Meimaridou et al., 2006). Purity of the mitochondrial preparation was confirmed by showing enrichment of succinate dehydrogenase (SDH) activity in the preparation.

2.6. Preparation of renal tissue lysates

Tissue lysates were prepared as described (Kiroycheva et al., 2000). In brief, the frozen kidney was homogenized in ice-cold homogenizing buffer containing 50 mM Tris pH 7.5, 20 mM NaCl, 1 mM EDTA, 0.5% NP-40, phosphatase inhibitors (100 mM NaF, 1 mM Na_3VO_4) and protease inhibitors cocktails. The homogenates were centrifuged at 10,000*g* for 10 min. Resultant supernatants were used to determine levels of GRP78, phospho-PERK and CHOP by western blot analysis.

2.7. Isolation of DNA from rat kidney tissue

DNA was isolated from snap-frozen rat kidney tissue using TRI reagent, according to the manufacturer's instructions.

2.8. Estimation of protein

The protein content of the cytosolic, nuclear and mitochondrial preparations and tissue lysates were determined, as described previously (Lowry et al., 1951).

2.9. Assessment of markers of apoptosis

2.9.1. DNA laddering

DNA that was isolated was electrophoresed at 80 V in a 1% agarose gel that contained 0.05% ethidium bromide, using Tris-acetate-EDTA (TAE) buffer (containing 40 mM Tris-acetate and 1 mM EDTA, pH 8.3) to look for DNA laddering, which was taken as evidence of DNA fragmentation. The separated bands were visualized and documented, using an AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA.).

2.9.2. Measurements of caspase activities

Activities of caspases-3 and -9 were determined as described previously (Yang et al., 2003), with some modifications. In brief, the kidney tissue samples were homogenized in lysis buffer