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The inhibitory effect of diphenyltin on gap junctional intercellular communication in HEK-293 cells is reduced by thioredoxin reductase 1

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ABSTRACT

Organotins display high biological activity and are toxic to animals and humans. Besides carcinogenic effects, they have been shown to have highly immunotoxic and/or neurotoxic activity; however, the molecular mechanism of their toxicity is not fully understood. The ability of chemicals to inhibit communication via gap junctions has been associated with their toxicological properties. The aim of this study was to determine whether diphenyltin (DPhT) affects the gap junctional intercellular communication (GJIC) and whether thioredoxin reductase (TrxR1) is involved in the regulation of this process.

We found that DPhT inhibits GJIC in HEK-293 cells. The inhibition of GJIC depends on the activation of PKC δ and is associated with the induction of Cx43 phosphorylation at Ser262. Moreover, we found that GJIC inhibited by DPhT in HEK-293 cells is fully re-established as a result of TrxR1 overexpression.

1. Introduction

Organotin compounds cause extensive contamination of the environment. Over the last several decades they have been utilized for a variety of industrial and agricultural applications including plastic stabilizers, anti-fouling agents, pesticides and fungicides, resulting in their accumulation in the environment and consequently in biological systems (Appel, 2004). Organotins display high biological activity and are toxic even at low concentrations. Besides carcinogenic effects, they have been shown to have highly neurotoxic, hepatotoxic, immunotoxic and/or nephrotoxic activity (Opacka and Sparrow, 1985; Costa, 1998; Nielsen and Strand, 2002; Appel, 2004). While the toxicological effects of organotin compounds are well documented, the possible mechanisms of their toxicity are not fully understood. At the cellular level, organotin compounds induce various membranous effects, including changes in membrane lipids, membrane rupture, blebbing, cell fusion, and erythrocyte haemolysis (Gray et al., 1987; Kleszczyńska et al., 1997). Organotin intoxication also resulted in reactive oxygen species production, cytoskeletal modifications and changes in the motile activity of several cell types (Gennari et al., 2000; Sroka et al., 2001, 2007). Recently, the effect of organotin compounds on the gap junctional intercellular communication (GJIC) has been suggested by Kishta et al. (2007).

GJIC is an important mechanism controlling cellular homeostasis, cell growth, differentiation, wound healing, but also pathologic processes such as tumorigenicity (Loewenstein, 1979; Richards et al., 2004; Czyz, 2008). Gap junctions comprised of connexins form trans-membrane channels connecting cytoplasms of adjacent cells and permitting the direct exchange of small metabolites and second messengers (Goodenough and Revel, 1971; Söhl and Willecke, 2004). Several extrinsic factors, including calcium ions, pH, transmembrane voltage and connexins phosphorylation affect channel permeability (Verselis et al., 1994; Peracchia, 2004). Phosphorylation of connexins appears to be implicated in the regulation of GJIC at several stages of the connexin life-cycle and provides a key mechanism regulating channel gating, as well as other aspects of connexin activity. It was shown that gap junction proteins could be direct targets for several kinases including protein kinase C (PKC), protein kinase A (PKA), tyrosine kinase Src, serine/threonine kinases MAPK and Cdc-2 kinase (Cruciani and Mikalsen, 2002).

The PKC family can be divided into three subgroups: conventional PKCs (α, βI, βII, γ), novel PKCs (δ, ε, η, θ), and atypical PKCs (ξ, λ/ι). Conventional PKC isoforms are stimulated by diacylglycerol and calcium. Novel PKC isoforms are also diacylglycerol stimulatable but calcium independent. Atypical PKC isoforms are stimulated by lipids but are not activated by calcium and diacylglycerol (Wicki

Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; DPhT, diphenyltin; GJIC, gap junctional intercellular communication; HEK-293 cells, human embryonic kidney cells; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; siRNA, small interfering RNA; Trx, thioredoxin; TrxR, Trx reductase; HEK-TrxR15 cells, HEK-293 cell line overexpressing TrxR1.

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and Niggli, 1999). Apart from classical regulation, PKC activity may also be controlled by redox mechanisms. It was reported that the oxidation of the regulatory domain of PKC causes enzyme activation, while reduction reverses this process (Domenicotti et al., 2003; Konishi et al., 1997; Korichneva et al., 2002). Since activators of PKC attenuate GJIC and increase connexin phosphorylation, PKC may provide a functional link between redox-dependent signalling pathways and the regulation of gap junctional communication.

One of the enzymes that is involved in the redox regulation of the activity of PKC is thioredoxin reductase (TrxR) (Gopalakrishna and Jaken, 2000). TrxR is a multifunctional selenoprotein which forms an oxidoreductase system, with thioredoxin (Trx) as a substrate and NADPH as a cofactor. Apart from protection against oxidative stress (Holmgren, 2000) and supplying reducing equivalents for the synthesis of deoxyribonucleotides (Laurent et al., 1964) the thioredoxin system is involved in a number of cellular processes, including redox regulation of transcription factors, and modulation of cell growth and apoptosis via the regulation of various kinases such as ASK-1 and PKC, and phosphatases such as PTEN and PTP1B (Gopalakrishna and Jaken, 2000; Saitoh et al., 1998; Lee et al., 2002; Rhee et al., 2005). Moreover, previously we reported that TrxR1is also involved in the regulation of migration of HEK-293 cells, induced by PKC activators, phorbol 12-myristate 13-acetate (PMA) and diphenyltin (DPhT) (Sroka et al., 2007).

Organotins accumulate in the kidney, and consequently can cause renal dysfunction (Opacka and Sparrow, 1985; Lin and Hsueh, 1993). Connexins, are widely distributed in the human kidney and play an important role in renal autoregulatory mechanisms and in the control of vasomotor responses (Wagner, 2008). Since phosphorylation of connexins is involved in the regulation of GJIC (Cruciani and Mikalsen, 2002) and it was demonstrated that organotins activate PKC (Pavlakovic et al., 1995; Sroka et al., 2007) we conducted this study to evaluate the effect of DPhT on the gap junctional coupling in human embryo kidney HEK-293 cell populations. Moreover, because PKC activity, apart from classical regulation, may also be controlled by redox mechanisms, and TrxR may regulate PKC activity (Gopalakrishna and Gundimeda, 2001), in the present experiments we used of HEK-293 cells, which stably overexpress TrxR1 (Nalvarte et al., 2004) to determine the role of TrxR1 in the regulation of gap junctional coupling of HEK-293 cells stimulated by DPhT.

2. Materials and methods

2.1. Materials

Chemicals were purchased from the following sources: diphenyltin, Dulbecco's modified Eagle's medium and F-12 nutrient mixture (DMEM/F-12), phorbol 12-myristate 13-acetate (PMA), protease inhibitor cocktail, rabbit polyclonal anti-cx43 antibody (Sigma); rabbit polyclonal anti-PKC δ antibody, rabbit polyclonal anti-p-S262-cx43 antibody (Santa Cruz Biotechnology Inc.); horseradish peroxidase-labeled goat anti-mouse IgG, horseradish peroxidase-labelled goat-anti-rabbit IgG, calcein AM, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes), Cy3-conjugated goat anti-mouse IgG (Dianova); fetal calf serum (Gibco, Invitrogen); FuGene6 Transfection Reagent (Roche), plasmid pKD-PKC δ-v6, pKD-NegCon-v1 (Upstate).

2.2. Cell culture

Stable human embryo kidney (HEK-293) cells which overexpress TrxR1 (HEK-TrxR15) were established as described previously (Nalvarte et al., 2004; Madeja et al., 2005). Control cells (HEK-IRES) were prepared by transfecting HEK-293 cells with the empty pIRES vector. Cells were cultured in DMEM/F-12 supplemented with 10% fetal calf serum and 100 μg/ml gentamicin. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Analysis of gap junctional coupling

GJIC was measured by double-labeling fluorescent dye transfer assay as described previously (Miekus et al., 2005). Homologous coupling was assessed for both HEK-IRES and HEK-TrxR15 cells. Cells were grown on plastic dishes to a density of about 70–100%. Then cells were incubated with or without 750 nM DPhT for 2 h. Donor cells were labeled with 5 μM calcein AM and 9 μM DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) for 30 min in PBS supplemented with glucose (1 mg/ml). Double-labeled donor cells were washed twice and plated on the acceptor cells at a 1:50 ratio and incubated for a further 2 h at 37 °C. In some experiments cells were preincubated with 5 mM N-acetyl-L-cysteine for 1 h or transfected with plasmid pKD-PKC δ-v6, as described below, followed by the addition of 750 nM DPhT. Calcein transfer from donor cells to recipient cells was evaluated using a Leica DM IRE2 microscope. Gap junctional coupling was quantified as the number of recipient cells that obtained calcein from one donor cell. At least 50 donor cells per coverslip were analyzed in three independent experiments performed for each experimental condition.

2.4. Transfection with the siRNA plasmid

Transfection of HEK-IRES cells with the plasmid pKD-PKC δ-v6 or pKD-NegCon-v1 was carried out according to the protocol supplied with the manufacturer's instructions (Upstate). Cells were grown for 72 h after transfection. The knock-down efficiency was tested by Western blot analysis from the whole-cell lysates as described previously (Keller et al., 2000; Sroka et al., 2007). Blots resulting from three independent Western blot assays were densitometrically assessed.

2.5. Western blotting

HEK-IRES and HEK-TrxR15 cells were incubated with or without 750 nM DPhT for 2 and 4 h and for the whole-cell lysates were treated as described previously (Keller et al., 2000). Cellular proteins (30 μg/lane) were then applied to 10% SDS–polyacrylamide gels, followed by transfer to nitrocellulose. Blots were exposed to polyclonal rabbit anti-cx43 antibody (1:5000) or polyclonal rabbit anti-p-(S262)-cx43 (1:500). The antibodies were then detected using horseradish peroxidase-labeled goat anti-rabbit (1:9000) IgG secondary antibodies with the help of an ECL Western detection system (Pierce, Rockford, IL). Blots resulting from three independent Western blot assays were densitometrically assessed.

2.6. Immunofluorescence

HEK-IRES and HEK-TrxR15 cells incubated with or without or 750 nM DPhT for 4 h were fixed with methanol/acetone (7:3) at −20 °C for 10 min and labeled with rabbit anti-cx43 antibody (1:200) for 1 h, followed by incubation with the secondary antibody, Cy3-conjugated goat anti-rabbit antibody (1:500) for 45 min. Cells were analysed with an epifluorescence Leica DM IRE2 microscope.

2.7. Statistical analysis

Statistical significance was determined using the nonparametric Mann–Whitney *U*-test with $p < 0.05$, considered to indicate significant differences.

3. Results

3.1. The effect of DPhT on the GJIC in HEK-293 cells

In a previous paper we showed that 750 nM DPhT activates PKC δ in HEK-293 cells (Sroka et al., 2007). Since it was demonstrated that GJIC is inhibited by PKC activator, PMA (Murray and Fitzgerald, 1979; Yotti et al., 1979) in the present paper we analysed the effect of DPhT on GJIC in HEK-293 cells. Moreover, because TrxR1 is involved in the regulation of PKC in these cells (Sroka et al., 2007) we also determined the effect of TrxR1 overexpression on the modulation of GJIC by DPhT. For this purpose we used HEK-293 cells, characterized by stable overexpression of TrxR1 (HEK-TrxR15 cells) and control HEK-IRES cells.

Experiments revealed that exposure of control HEK-IRES cells to 750 nM DPhT for 4 h resulted in the complete inhibition of GJIC illustrated by lack of calcein diffusion (green) between double-labeled donor (red) and recipient cells (Fig. 1A and B). Interestingly, the dye transfer in TrxR1 overexpressing HEK-TrxR15 cells (Fig. 1C and D), incubated in the presence of 750 nM DPhT was only slightly decreased (to 76% of the control). The average values of the coupling index (C_r), i.e. the number of recipient cells that obtained calcein from one donor cell for HEK-IRES and HEK-TrxR15 cells are shown in Fig. 2. These results suggest that DPhT affects GJIC in HEK-IRES