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Transient disruption of liver gap junctional intercellular communication and induction of apoptosis after administration of 1,4-bis[2-(3,5 dichloropyridyloxy)]benzene in mice

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ABSTRACT

Gap junctional intercellular communication (GJIC) and connexin expression (Cx26 and Cx32) in mouse liver were studied after administration of 4-bis[2-(3,5 dichloropyridyloxy)]benzene (TCPOBOP), a phenobarbital-like enzyme inducer. Female C57Bl/6 mice were administered TCPOBOP (5.8 mg/kg BW) and euthanized 0, 24, 48 and 72 hours later. Liver samples were snap frozen, or fixed in formalin, or submitted to GJIC analysis. The proliferating cell nuclear antigen (PCNA) immunohistochemistry and the Western blotting for Cx26 and Cx32 were performed. After 48 and 72 h of drug administration the liver-to-body weight ratio was increased 70% and 117% ($p<0.0001$), respectively. There were temporal-dependent alterations in liver histopathology and a significant increase in cell proliferation was noted after 48 h and sustained after 72 h, though to a lesser extent ($p<0.0001$). In addition, TCPOBOP administration induced apoptosis, which appeared to be time-dependent showing statistical significance only after 72 h ($p<0.0001$). Interestingly, a transient disruption by nearly 50% of GJIC capacity was detected after 48 h of drug ingestion, which recovered after 72 h ($p = 0.003$). These GJIC changes were due to altered levels of Cx26 and Cx32 in the livers of TCPOBOP-treated mice. We concluded that a single administration of TCPOBOP transiently disrupted the levels of GJIC due to decreased expression of connexins and increased apoptotic cell death in mouse liver.

Introduction

Gap junctional intercellular communication (GJIC) is most acknowledged for its role in the exchange of molecules smaller than 1.5 KDa between adjacent cells. The GJIC are formed when two adjacent transmembranous small channels from each cell interact with each other and connect. These channels, known as connexons, are formed by the oligomerization of six proteins, the connexins, a protein family with more than 20 different types in mammals (Willecke et al., 2002) showing developmental and tissue-specific expression patterns. Impaired levels and function of these proteins are demonstrated in several different pathological conditions such as non-syndromic deafness (Kelsell et al., 1997), peripheral nerve disorders, such as Charcot-Marie-Tooth disease (Bergoffen et al., 1993) and cancer (Yamasaki, 1996).

Several non-genotoxic carcinogens inhibit the connexin-mediated GJIC in vivo and in vitro (Yamasaki, 1996), including, for example, carbon tetrachloride (CCl4), p,p-dichlorodiphenyltrichloroethane (DDT), Wy-14,643 (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid) (Tateno et al., 1994; Chipman et al., 2003) and phenobarbital (PB) (Pereira et al., 1986). Surprisingly, 1,4-bis[2-(3,5 dichloropyridyloxy)]benzene (TCPOBOP) still has not been evaluated as an inhibitor of connexins or GJIC, even though being functionally related to phenobarbital. The TCPOBOP is 650 times as potent as phenobarbital regarding the induction of microsomal epoxide hydrolase activity (Poland et al., 1980). Also, both drugs induced pronounced effects on liver weight, proliferation of the endoplasmic reticulum and cytosolic glutathione S-transferase activity (Poland et al., 1980). In addition, it was already shown that the pleiotropic effects of TCPOBOP, but not phenobarbital, occurred because the drug is an agonist ligand of the constitutive androstane receptor (CAR), which belongs to the family of the xenobiotic nuclear receptors (Tzameli et al., 2000). Diwan et al. (1992) showed that TCPOBOP was a potent inducer and tumor promoter in mice, but was negligibly effective as either an inducer or a promoter in F344 rats at even a 10-fold higher dosage. Later, the same authors demonstrated that TCPOBOP at maximal CYP2B induction doses exhibited a strong promoting activity for both liver and thyroid of rats (Diwan et al., 1996). Following these evidences, it was shown that CAR is essential for

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liver tumor promotion by PB in mice (Yamamoto et al., 2004) and later for TCPOBOP (Huang et al., 2005).

Thus, the intention of the present study was to show the time and the course of GJIC and connexins levels of mouse liver after a single administration of TCPOBOP, a known CAR ligand.

Material and methods

Chemicals

The TCPOBOP was synthesized as previously described (Poland et al., 1980) and was kindly provided by Ms. Croizy (Paris, France). Corn oil was from Mazolla® (Sao Paulo, Brazil). Lucifer yellow, rhodamine, 3′3-diaminobenzidine (DAB), trizma, horseradish peroxidase anti-rabbit and anti-mouse IgG antibodies and B-actin antibody were obtained from Sigma Chemical Co. (Saint Louis, USA). The proliferating cell nuclear antigen (PCNA) antibody and skimmed milk were from Dako (Denmark). Bio-Rad Protein Assay reagent was purchased from Bio-Rad Labs (Hercules, USA). Anti-Cx26 and Anti-Cx32 rabbit polyclonal antibodies were from Zymed Laboratories (San Francisco, USA). All other reagents were analytical grade.

Animals

Forty-eight 3-month-old female C57Bl/6 mice were bred and maintained at the animal facility of the Department of Pathology at the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (FMVZ-USP) on a 12 h light/12 h dark cycle (lights on at 7 AM) under controlled conditions of temperature ($20 \pm 4\,^{\circ}C$), and relative humidity ($55 \pm 10\%$). Mice had *ad libitum* access to a standard diet (NUVILAB-CR1®, Nuvital Nutrientes Ltd. A) and filtered water. All procedures using animals were performed following "Principles of laboratory animal care" (NIH publication no. 85-23, revised in 1985) and were reviewed and approved by the Bioethics Committee of the FMVZ-USP (process number 953/2006). Female mice were chosen because they are more susceptible to TCPOBOP effects than males (Ledda-Columbano et al., 2003). The C57Bl/6 breed was chosen according to a previous experiment from our group (Dagli et al., 2004).

Experimental protocol

The animals received 5.8 mg/kg of TCPOBOP diluted in corn oil through gavage and were killed immediately, or 24, 48 and 72 h after TCPOBOP (12 animals per time-point) as previously published (Dagli et al., 2004). All animals were weighted before TCPOBOP- and once a day after TCPOBOP-administration. Following euthanasia (thiopental 250 mg/kg, I.P), the livers were harvested, weighted and sampled for histological analysis, or snap frozen in liquid nitrogen and conserved at $-80\,^{\circ}C$ until analysis.

Histopathological analysis

Representative slices from each lobe were fixed in 10% formalin for 96 h, dehydrated, processed, embedded in paraffin wax and stained with hematoxylin and eosin.

Immunohistochemistry for PCNA

Detection of the proliferating cell nuclear antigen (PC 10, diluted 1:3200) was performed in representative liver sections (5 μm) for each animal as previously described (Fukumasu et al., 2008). Briefly, liver sections were de-paraffinized and re-hydrated with consecutive baths with xylene and ethanol at different concentrations. The endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 5 min. Later, non-specific protein binding sites were blocked with 5% skim milk for 2 h and slides were subjected to PCNA antibody overnight at $4\,^{\circ}C$. Slides were then exposed to streptavidin–biotin–peroxidase method according to the manufacturer (LSAB) and PCNA protein was detected after DAB staining. Haematoxylin was used to counterstain the slides.

Positive PCNA cells have their nucleus colored brown due to DAB deposition. In the present study, at least 1000 cells were counted per animal. The proliferation index was obtained by dividing the number of brown nuclear stained cells by the total number of cells counted for each animal and multiplied per 100.

Fluorescent detection of apoptotic bodies

Apoptotic index were analyzed as previously described (Stinchcombe et al., 1995) with minor modifications (Fukumasu et al., 2008). The presence of hepatocyte apoptotic bodies (AB) were evaluated through fluorescence microscopy using a Nikon microscope (E-800, Tokyo, Japan) equipped with an epi-fluorescence unit. This method is based on the strong eosin fluorescence of AB in H&E-stained tissues detected under UV light (465–495 nm). Identification of AB was confirmed by switching the microscope system from fluorescent to transmitted light and using morphological criteria established by Kerr et al. (1972). ABs were represented by acidophilic bodies with fragmentation or lack of chromatin accompanied by cytoplasmic condensation and/or fragmentation. If single cells or clusters of directly neighboring cells contained multiple AB, these were assumed to be derived from the same apoptotic cell and were counted as only one event. Four randomly chosen fields of representative liver sections were taken for each animal and at least 3000 cells were counted. Apoptosis index (AI) was expressed as the number of apoptotic hepatocytes per 1000 hepatocytes.

Gap junction intercellular communication analysis

The incision loading dye transfer was performed accordingly with Sai et al. (2000) and slightly modified by da Silva et al. (2009). This method is based on the Lucifer yellow (LY-457 Da) dye ability of diffusing through adjacent cells by gap junctions due to its size (less than 1 KDa). On the other hand, rhodamine (RhD) will not diffuse because it is greater than 1 KDa. Briefly, hepatic tissue were excised, rinsed in PBS and put in the external side of a glass tube to allow the dye mixture stay in the incisions. The mixture of fluorescent dyes containing 0.5% LY and 0.5% RhD in PBS was dropped onto the tissue surface. Three to four incisions (7–8 mm long) were made on the surface of each specimen with a sharp blade (1 mm depth). Additional dye mixture was put into the incisions and kept for 5 min at room temperature. After incubation, the tissue was rinsed in PBS three times and fixed in 10% buffered formalin overnight. Slices were quickly rinsed in water and then routinely processed for embedding in paraffin. Sections (5 μm) for GJIC analysis were prepared by cutting the paraffin block perpendicular to the incision line. Areas stained with LY alone or with RhD were detected by fluorescence emission using a microscope (Nikon Eclipse-800, Japan) equipped with an epi-fluorescence unit. The net area stained with LY alone and the length of incision was quantified with an electronic pen and the software calculated automatically (Image Pro-Plus, version 4.5, Media Cybernetics). At least three of the incision sites per specimen were randomly chosen for analysis and the mean value