J. Pharm. Pharmacol. 1992, 44: 609–611
Communicated October 30, 1991

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Effect of exogenous copper on lipid peroxidation in rat hepatocytes. Possible involvement of protein kinase C

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Abstract—We have investigated the direct effect of copper on malondialdehyde formation in rat isolated hepatocytes. Copper was found to decrease the cell viability with concomitant production of malondialdehyde in a time related manner. In addition the protein kinase C activator, PMA, was found to have a synergistic effect with copper on rat hepatocytes. These results indicate that protein kinase C may be important in mediating hepatotoxicity after exposure to copper.

Copper is hepatotoxic in man (Chuttani et al 1965), and chronic accumulation results from inherited defects in copper metabolism (Popper et al 1979). The exact mechanism of copper-induced toxicity remains unclear. However, it has been reported that copper initiates lipid peroxidation by participating in the Haber–Weiss reaction (Aust et al 1985) and copper with some other oxidants has been shown to produce lipid peroxidation in cultured hepatocytes (Von Reucker et al 1989).

The aim of our study was to investigate the effect of copper on malondialdehyde production and the involvement of protein kinase C in copper-induced lipid peroxidation. The effect of a protein kinase inhibitor on copper-induced lipid peroxidation was also studied in order to confirm the involvement of protein kinase C in copper-induced lipid peroxidation.

Materials and methods

Hepatocytes were isolated from male Wistar rats, 150–200 g, by the collagenase (Sigma) perfusion method described by Moldeus et al (1973).

Cells (10^6 mL^{-1}) were incubated in Krebs–Henseleit buffer pH 7·4 supplemented with 2% bovine serum albumin and 1% D-glucose at 37°C under an atmosphere of 95% O_2–5% CO_2. Hepatocytes were exposed to different concentrations of copper in two sets of experiments. The concentrations were 0 (saline), 10, 100, 500, 1000, 2000 μM copper ($CuCl_2$) (experiment 1) and 200, 400, 800, 1600 μM (experiment 2) for a maximum of 30 or 60 min. The viability of the cells was checked at different time intervals by the trypan blue exclusion test after the addition of $CuCl_2$. To estimate the degree of Cu^{2+} uptake into the hepatocyte during the 60 min incubation, 1 mL of cell suspension was washed twice in Krebs–Henseleit buffer and the pellet of hepatocytes obtained was stored at −20°C and analysed for copper by atomic absorption spectroscopy within two weeks. Hepatocyte Cu^{2+} content was expressed as μg/10^6 cells.

Lipid peroxidation was measured as malondialdehyde formed, the end product of peroxidized fatty acid by thiobarbituric acid (Okawa et al 1979).

Results

Hepatocytes accumulated Cu^{2+} in a time-dependent manner (Fig. 1).

The viability of hepatocytes decreased with varying concentrations of Cu^{2+} (Fig. 2). Concomitantly malondialdehyde production increased, with increasing $CuCl_2$ concentration up to 2000 μM Cu^{2+} (Fig. 3).

To investigate further the effect of Cu^{2+} on malondialdehyde production, $CuCl_2$ concentration and exposure time were increased and malondialdehyde was measured at 30 and 60 min (Fig. 4).

To evaluate the involvement of protein kinase C in copper-induced lipid peroxidation, hepatocytes were incubated with PMA (4-β-phorbol-12-myristate-13-acetate) (Sigma) and the

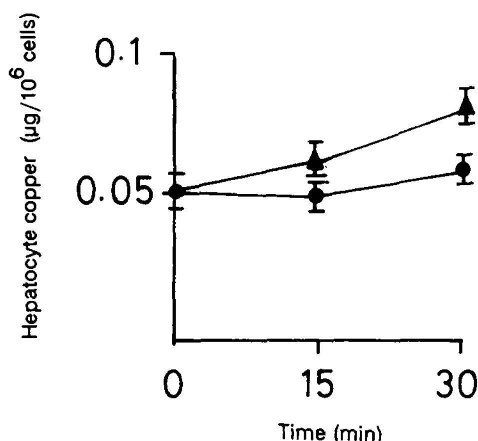

FIG. 1. Copper content of isolated hepatocytes after incubation with $CuCl_2$ for 30 min. The results are given as mean ± s.e.m. (n = 3). ●, 500 μM; ▲, 800 μM.

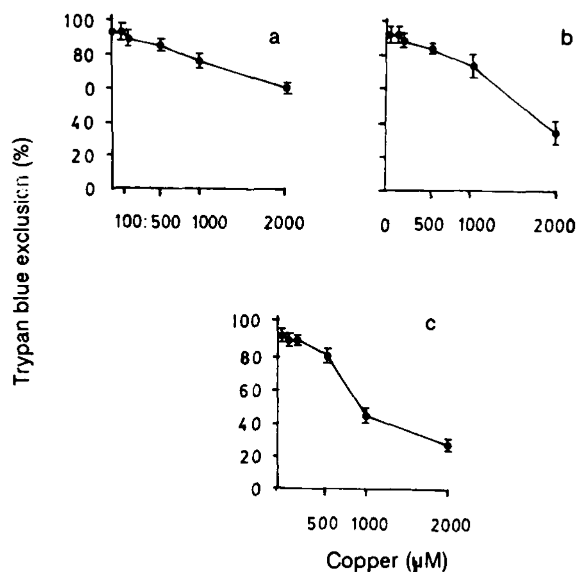

FIG. 2. Trypan blue exclusion of isolated hepatocytes after incubation with varying concentrations of $CuCl_2$: (a) t = 15 min; (b) t = 30 min; (c) t = 60 min. Results are mean ± s.e.m. (n = 3).

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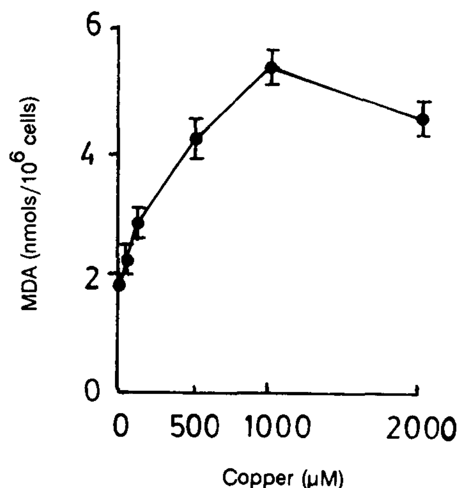

FIG. 3. Malondialdehyde (MDA) production (nmol/10^6 cells) by isolated hepatocytes with different concentrations of $CuCl_2$. Incubation of hepatocytes with indicated concentrations of $CuCl_2$ was carried out for 30 min. Mean ± s.d. represent 5 separate preparations.

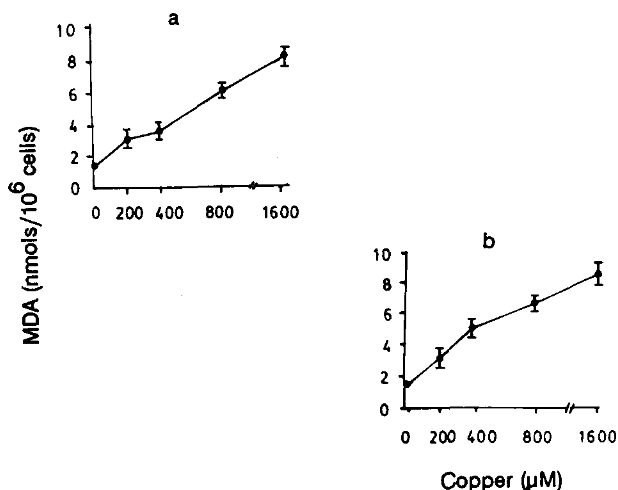

FIG. 4. Effect of increased concentrations of copper on malondialdehyde (MDA) production after 30 min incubation (a) and 60 min incubation (b). Mean ± s.d. represent 5 separate preparations.

protein kinase C antagonist, H-7 (1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine). PMA was found to have a synergistic effect with copper on malondialdehyde formation while H-7 significantly reduced malondialdehyde production (Fig. 5).

Discussion

The potential of Cu^{2+} to induce peroxidative damage was investigated in-vitro. The results of our study demonstrated that exposure of rat isolated hepatocytes to $CuCl_2$ produced toxic changes, manifested as an increased LDH release (data not shown) and a resultant decrease in cell viability. The changes were associated with an increase in the production of malondialdehyde, suggesting a direct induction of lipid peroxidation by the metal.

The increase in malondialdehyde production in response to Cu^{2+} was strongly suppressed by H-7, a potent inhibitor of protein kinase C (Hicaka et al 1984; Grove & Mastro 1988). The inhibitory action of this agent might be due to its ability to

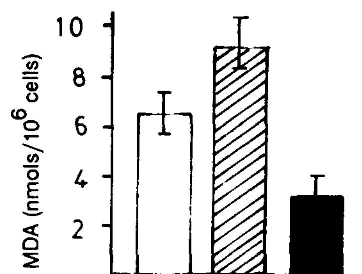

FIG. 5. Effect of a protein kinase C antagonist (H-7) and agonist (PMA) on malondialdehyde (MDA) production. H-7 (100 μM) could bring a significant decrease in MDA production $P < 0.01$ as compared with MDA produced by 800 μM $CuCl_2$. A significant increase in MDA production $P < 0.005$ is observed by PMA (1 μM) as compared with MDA produced by 400 μM $CuCl_2$ after 30 min exposure. Results are expressed as mean ± s.d. (n = 5). □, $CuCl_2$ (800 μM); ■, $CuCl_2$ + PMA; ■, $CuCl_2$ + H-7.

scavenge oxygen free radicals or due to its action as a protein kinase C inhibitor. Recent studies on H-7 have clearly shown that H-7 does not scavenge oxygen free radicals (Johnson et al 1989), indicating that malondialdehyde production is governed by a protein kinase C mechanism. The finding that PMA, a known activator of protein kinase C, could significantly stimulate malondialdehyde production induced by Cu^{2+}, lends strong support to this interpretation. An important question arises from these arguments: is the protein kinase C activated by oxygen free radicals generated in response to Cu^{2+} or is it activated by the metal itself? It is well-documented that copper can directly interact with molecular oxygen to initiate the production of a series of toxic oxygen species (Lindquist 1968; Halliwell 1989). The mechanism by which protein kinase C is activated by oxygen radicals is not clear. In-vitro studies have shown that this second messenger is activated by the initial products of lipid peroxidation (O'Brian et al 1988). It has also been suggested that diacylglycerol, the inherent protein kinase C activator, is induced by oxygen free radicals (McCord 1985; Nishizuka 1986). It is possible that an increase in intracellular Ca^{2+}, as a consequence of copper-induced hepatic damage (Halliwell & Gutteridge 1984) could bring about an indirect activation of protein kinase C via the breakdown of membrane phosphoinositides (Nishizuka 1986). Although an indirect activation of protein kinase C mediated by oxygen free radicals or otherwise, is strongly suggested, a direct activation of protein kinase C cannot be ruled out, particularly in light of the study by Csermely et al (1988) indicating a direct activation of protein kinase C by a divalent metal ion, zinc. It is worth mentioning that the specific metal chelator, used in this study, also chelates small amounts of copper ions. This nevertheless needs further investigation.

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J. Pharm. Pharmacol. 1992, 44: 611–614
Communicated December 6, 1991

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Induction of propranolol metabolism in the Hep G2 human hepatoma cell line

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Abstract—Metabolism of propranolol by the human hepatoma cell line Hep G2 was studied. Although metabolism qualitatively was similar to that in-vivo, the P450-mediated *N*-desisopropylation clearly predominated. Pretreatment of cells with 3-methylcholanthrene increased the activity of this pathway 14-fold, whereas phenobarbitone had no effect. This is similar to the pathway-selective inductive response observed for cigarette smoking in-vivo. As in-vivo, secondary metabolism of *N*-desisopropylpropranolol was extensive. This could, however, be completely blocked by 0·1 μM clorgyline, a potent MAO type A inhibitor. As in human liver microsomes, the stereochemistry of propranolol metabolism demonstrated a preference for the *R*(+)-enantiomer. These observations emphasize the usefulness of the Hep G2 cell line as a model of man.

Propranolol is commonly used as a model compound in in-vivo and in-vitro human drug metabolism studies. A wide range of environmental and genetic factors is known to affect propranolol's metabolic clearance (Lennard et al 1984; Raghuram et al 1984; Walle et al 1985a; Ward et al 1989). Two such factors of importance for drug disposition in general are sex differences and cigarette smoking, both of which have selective effects on the cytochrome P450-mediated metabolism of propranolol. Thus, the metabolic clearance of propranolol is higher in men than in women (Walle et al 1989) and also higher in cigarette smokers compared with nonsmokers (Walle et al 1987), mainly due to higher clearance through side-chain oxidation (Fig. 1).

The objective of the present investigation was to examine the human Hep G2 cell line (Dawson et al 1985; Sassa et al 1987; Grant et al 1988; Doostdar et al 1990; Fischer & Wiebel 1990) as a potential in-vitro hepatic model for the human metabolism of propranolol, in particular side-chain oxidation, and its regulation by enzyme inducers and other factors.

Materials and methods

Materials. Hep G2 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and Williams' medium E was from Gibco BRL (Grand Island, NY, USA). Foetal calf serum, propranolol HCl, phenobarbitone sodium salt, clorgyline and pargyline were purchased from Sigma Chemical Co (St Louis, MO, USA). (±)-[4-^{3}H]Propranolol HCl (sp. ac., 25 Ci mmol^{-1}) was from Amersham Corp. (Arlington Heights, IL, USA), 3-methylcholanthrene from Eastman (Rochester, NY, USA) and (−)-menthyl chloroformate (MCF) from Aldrich Chemical Co (Milwaukee, WI, USA). 4-Hydroxypropranolol (4HOP)(Fig. 1), 5-hydroxypropranolol (5HOP), *N*-desisopropylpropranolol (DIP), propranolol glycol (Glycol) and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) were synthesized in our laboratories (Nimura et al 1980; Oatis et al 1981; Bargar et al 1983). α-Naphthoxylactic acid (NLA) was a gift from ICI Ltd (Wilmslow, UK). All solvents were of HPLC grade from Burdick & Jackson (Muskegon, MI, USA).

Culture of Hep G2 cells. Cells were grown in a humidified atmosphere of 5% CO_2 in air in Williams' medium E supplemented with 10% (v/v) foetal calf serum, 100 int. units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Doostdar et al 1990). The cells were split 1:3 every 7 days and used for experiments on day 11 after passage (Doostdar et al 1990). The medium was renewed routinely 3, 6 and 10 days after passage. In the induction studies, 2 μM 3-methylcholanthrene (in dimethyl sulphoxide) or 2 mM phenobarbitone was added to the cells 24 h before experiments. These concentrations are inductive in the Hep G2 cells (Grant et al 1988; Labruzzo et al 1989). The dimethylsulphoxide, final concentration 0·2% in the medium, had no effect on metabolism.

Incubations. Washed cells were scraped off the culture into tubes with 100 mM phosphate buffer, pH 7·6 (1 mL 100 mm dish), and pressurized with nitrogen in a cell disruption bomb (1000 psi for 10 min). To 0·5 mL of disrupted cell homogenate was added an NADPH-generating system (0·5 mM NADP, 10 mM glucose-6-phosphate, 5·2 mM magnesium chloride and 2 int. units mL^{-1} glucose-6-phosphate dehydrogenase) in buffer for a total volume of 1·0 mL. Incubations were carried out for 30 min at 37°C in a

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