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The Role of Protein Kinase C in the Increased Generation in Isolated Rat Hepatocytes of the Hydroxyl Radical by Puromycin Aminonucleoside

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Puromycin aminonucleoside (PAN) has been known to induce proteinuria. The increased generation of reactive oxygen species (ROS) has been implicated in this toxicity of PAN. We have reported that PAN increases the synthesis of methylguanidine (MG) and creatol which are the products of the reaction of creatinine and the hydroxyl radical in isolated rat hepatocytes. However, the mechanism for the increased ROS induced by PAN is still unclear. In this paper, we investigate the role of protein kinase C (PKC) on the PAN induced reactive oxygen generation in isolated rat hepatocytes. Isolated hepatocytes were incubated in Krebs–Henseleit bicarbonate buffer containing 3% BSA, 16.6 mM creatinine and tested reagents. MG and creatol were determined by high-performance liquid chromatography using 9,10-phenanthrenequinone for the post-labeling. PAN increased MG and creatol synthesis in isolated rat hepatocytes by 60%. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), a PKC inhibitor, at 10 and 100 μM significantly inhibited MG and creatol synthesis with or without PAN. The inhibition rate is dose dependent from 10 to 100 μM. H1004, a reagent used as control for H-7, did not affect (at 10 μM) or increased little (at 100 μM) the synthesis of MG and creatol. Ro31-8425, a potent PKC inhibitor, significantly inhibited (at 10 μM) MG synthesis in the presence of PAN. PKC in the membrane fraction, a marker of PKC activation, increased over the initial concentration by a factor of 1.65-fold at 60 min incubation and 2.16-fold at 120 min with PAN, while it changed little without PAN. These results indicate that PAN activates PKC resulting in increased hydroxyl radical generation in isolated rat hepatocytes.

Keywords: Puromycin aminonucleoside, protein kinase C, reactive oxygen, hepatocyte

INTRODUCTION

Puromycin aminonucleoside (PAN) has been known to induce heavy proteinuria in rats following a single injection[1] while repeated injections of small amounts of PAN result in an increase in the glomerular mesangial matrix resembling the

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features of focal glomerular sclerosis, an intractable nephrosis.[2] Therefore, many investigators have used this reagent to create animal models for the study of nephrosis. Recently, ROS has been implicated as the cause of this toxicity induced by PAN. This is based upon the observation that ROS scavengers have a beneficial effect on proteinuria[3] as well as the finding of increased lipid oxidation in PAN-treated rats.[4] We have reported an increase in the generation of hydroxyl radical, the most reactive ROS, in the presence of PAN. This is indicated by an increased synthesis of methylguanidine (MG) and creatol in isolated rat hepatocytes within a few hours of the addition of PAN.[5,6] Since we showed that MG, a uremic toxin, is formed from creatinine through creatol, a hydroxyl radical adduct of creatinine,[5–10] it has become apparent that the synthetic rate of MG and/or creatol from a certain level of creatinine can be used as a marker for the rate of synthesis of the hydroxyl radical.[11,12]

It is well known that activation of protein kinase C (PKC) increases reactive oxygen generation in inflammatory cells.[13] However, in tissue cells such as hepatocytes, the role for protein kinase C on the generation of reactive oxygen is still unclear because of the difficulties in measuring reactive oxygen in tissue cells.

In this paper, we investigate the effect of a PKC inhibitor on hydroxyl radical generation and also the translocation of PKC as a marker for the activation of PKC in cells[14] to determine the role of PKC in the increased hydroxyl radical generation by PAN.

MATERIALS AND METHODS

Materials

Aminonucleoside of puromycin was purchased from Sigma Chemical Co., St. Louis. 1-(5-lsoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), a protein kinase inhibitor and N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). H-7 and HA1004 were dissolved at 10 mM in distilled water and kept as a stock solution. Ro31-8425 (Calbiochem) was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and kept as a stock solution. The creatol standard was kindly donated by Dr. Ienaga of the Nippon Zoki Pharmaceutical Co. Ltd. Monoclonal antibodies specific against α, β and γ isozymes were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). The cell lysate for the PKC standard was purchased from the Transduction Laboratory (Lexington, KY, USA).

Preparation of Isolated Rat Hepatocytes

Male Wistar rats weighing 300–350 g were used in all experiments. The rats were allowed free access to water and laboratory chow containing 25% protein. Isolated hepatocytes were prepared essentially according to the method of Berry and Friend[15] as described previously.[16,17] We calculated that 9.8×10^7 cells correspond to 1 g of wet hepatocytes.

Incubation of Cells for MG and Creatol Synthesis

Hepatocytes were incubated in 6 ml of Krebs–Henseleit bicarbonate buffer containing 3% bovine serum albumin, 10 mM sodium lactate and 16.6 mM creatinine and indicated substances. The incubation mixture was shaken at 60 cycles/min in a 30-ml conical flask with a rubber cap under 95% oxygen and 5% carbon dioxide at 37°C. Equilibration of the buffer was repeated every hour. Incubation was arrested by the addition of 0.6 ml of 100% (w/v) trichloroacetic acid.

Determination of MG and Creatol

After sonication, the supernatant of cells and medium was obtained by centrifugation at 1700× g for 15 min at 0°C, and 0.2 ml of the extract was used for MG and creatol measurements. MG was determined by high-performance liquid

chromatographic analysis using 9,10-phenanthrenequinone for the post-labeling method as described previously.[18] Dimethyl formamide for fluorometrical use was purchased from Wako, Co., Japan. Creatol was separated by a cation exchange resin column larger than that for MG analysis and was converted to MG by heating at 125°C under strong alkaline condition using the HPLC system modified from the MG determination apparatus.[19]

Subcellular Fractionation of PKC from Isolated Hepatocytes

Hepatocytes (1.5 g wet cells) were incubated in 35 ml of the same buffer as used for MG and creatol synthesis with or without PAN. An aliquot (5 ml) of the solution was taken at 0, 15, 30, 60 and 120 min and the cells were collected by centrifugation. The collected cells were homogenized with a Dounce homogenizer (100 strokes) in 2 ml of 50 mM Tris–HCl pH 7.6 containing 0.25 M sucrose, 2 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride and 0.1 mM leupeptin. After an initial centrifugation (600× *g* for 10 min) to remove nuclei, the supernatant was centrifuged at 12 000× *g* for 10 min to remove mitochondria and the supernatants were separated into cytoplasmic and insoluble fractions by centrifugation (105 000× *g* for 45 min). The insoluble fractions (membrane fraction) were homogenized in 200 ml of the buffer used for homogenization and were kept on ice for 30 min after the addition of 1% Triton X-100 for solubilization. The cytoplasmic and the insoluble fractions were boiled for 5 min in the presence of 2% sodium dodecyl sulfate (SDS), and both fractions were kept at −80°C. Protein in both fractions was determined by the bicinchoninic acid protein assay method.[20]

Determination of PKC

Both fractions were boiled for 3 min with 1% β-mercaptoethanol, 5% glycerol and 0.006% bromophenol blue. Proteins (110 μg protein) in the above two fractions were separated by electrophoresis on 8% SDS-polyacrylamide gels (14 × 14 cm). As a positive control of PKC (1 μg protein), rat brain lysate obtained from Transduction Laboratories was used. The proteins were transferred to nitrocellulose filters. Immunoreactive proteins were detected using the mixture of antibodies specific against α, β and γ isozymes. These were diluted 100 times and mixed (1:1:1). Enhanced chemiluminescence (ECL) western blotting detection reagents (RPN 2106, Amersham Pharmacia Biotech, NJ, USA) were used for detecting PKCs. Two sets of the films were prepared, one for the picture and another for densitometry. The bands of PKCs were quantified by densitometry using the films that were exposed to less chemiluminescence to obtain more accurate quantification.

Statistics

Significance of experimental results between two groups was evaluated using the unpaired student's *t*-test. For comparisons involving more than two groups, ANOVA was applied. Results were expressed as mean ± SE. The differences were considered statistically significant when the calculated p value was less than 5%.

RESULTS

Effect of PKC Inhibitors on MG Biosynthesis in Isolated Rat Hepatocytes

MG synthesis in isolated rat hepatocytes without PAN was 120 nmol/g wet cells/4 h. PAN increased MG synthesis from 120 to 165 nmol/g wet cells /4 h. H-7, a PKC inhibitor,[21] at 10 and 100 μM significantly inhibited MG synthesis by 26% and 37% in the absence of PAN and by 21% and 50% in the presence of PAN (Figure 1). HA1004 is the weakest PKC inhibitor among the isoquinolinesulfonamide derivatives,[22] and is useful as a control for H-7. HA1004 did not inhibit and, in fact, increased MG synthesis (Figure 1).

The effect of Ro31-8425,[23] a more specific and potent PKC inhibitor, on MG synthesis in isolated rat hepatocytes was investigated. Ro31-8425 should be dissolved in DMSO, a hydroxyl radical scavenger that inhibits MG synthesis. To correct this inhibition, a corresponding amount of DMSO in Ro31-8425 solution was added to the incubation medium for control. The corrected value for MG synthesis without PAN using 1 or 10 μM Ro31-8425 was 16.0 ± 2.4 and 13.0 ± 1.5 nmol/g wet cells/h (mean ± SE, $n = 5$) and 1 and 10 μM Ro31-8425 inhibited MG synthesis by 6% and 35%, respectively. The corrected value for MG synthesis with PAN using 1 or 10 μM Ro31-8425 was 27.8 ± 1.7 and 23.9 ± 0.95 nmol/g wet cells/h (mean ± SE, $n = 5$), and 1 and 10 μM Ro31-8425 inhibited MG synthesis by 10% and 24%, respectively. Ro31-8425 inhibited MG synthesis under all conditions and statistical significance was observed at 10 μM in the presence of PAN.

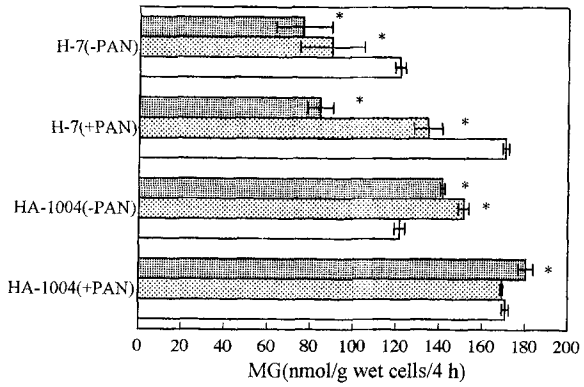

FIGURE 1 Inhibition of MG biosynthesis in isolated rat hepatocytes by H-7. Isolated hepatocytes (0.19 g wet cells) were incubated for 4 h as described in Materials and Methods section. ▭ represents without H-7, ▨ represents 10 μM H-7, ▩ represents 100 μM H-7. Each column represents mean of 5 incubations. Bars indicate tbe standard error. * represents p less than 0.05 vs. control value.

Time Course of Inhibition of MG and Creatol Synthesis by H-7

The time course of MG and creatol synthesis in the presence of 100 μM H-7 is shown in Figure 2. MG synthesis increased in proportion to the incubation period and was further increased by PAN. H-7 decreased MG synthesis in the presence or absence of PAN up to 6 h (Figure 2(a)). Creatol

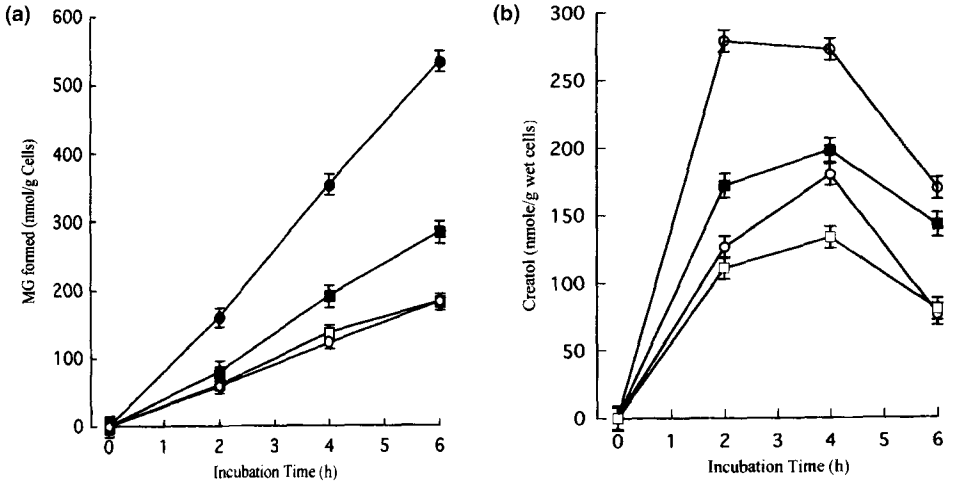

FIGURE 2 Time course of inhibition of MG and creatol synthesis by 100 μM H-7 in the presence or absence of PAN. Isolated hepatocytes (0.1 g wet cells) were incubated as described in Materials and Methods. MG synthesis is shown in Figure 2(a) and the amount of creatol found is shown in Figure 2(b). ● indicates with PAN, ○ indicates PAN plus H-7, ■ indicates without PAN, □ indicates PAN plus H-7. Each point represents the mean of duplicate incubations. Bars indicate the range of each determination.

levels are increased by PAN at 2 h and declined thereafter because of the conversion of creatol to MG. H-7 decreased creatol levels throughout the incubation period with or without PAN (Figure 2(b)).

Effect of the Concentration of H-7 on MG and Creatol Synthesis in Isolated Rat Hepatocytes

H-7 at concentrations ranging from 1 to 100 μM was investigated in the hepatocyte system. At concentrations from 10 to 100 μM it inhibited MG synthesis (Figure 3(a)) and decreased creatol levels (Figure 3(b)) significantly after 4 h and the inhibition rate in the presence of PAN was higher than in the absence of PAN (Figures 1 and 2).

Activation of PKC by PAN in Isolated Rat Hepatocytes with Increased Synthesis of Creatol

The translocation of PKC from the cytoplasmic fraction to the membrane fraction has been reported as one of the most reliable indicators of the activation of the conventional type of PKC in cells.[14,24,25] PKC detected by the western blotting method is shown in Figure 4.

PKC in the membrane fraction, a marker of PKC activation, were determined after 15, 30, 60 and 120 min incubations with or without 1.9 mM PAN. PKC in the membrane fraction with PAN after 15, 30, 60 and 120 min incubation increased 0.85, 1.1, 1.75 and 2.16-fold over that at 0 time and those without PAN were increased 1, 1.35, 1.25 and 1.12-fold over that at time 0 (Figure 5(a)). Thus the PKC in the membrane fraction markedly increased after 60 and 120 min incubation with PAN, while PKC in the membrane fraction without PAN changed little. The ratios of PKC in the membrane fraction with PAN to that without PAN after 15, 30, 60 and 120 min incubation are 0.84, 0.79, 1.4 and 1.93, respectively (Figure 5(a)). The amounts of PKC in the cytosol fractions are shown in Figure 5(b). The amount of PKC in the cytosol fraction did not differ much from that in the membrane fraction when expressed per unit of protein. However, the amount of protein in the cytosol fraction is huge compared to that in the membrane fraction. Therefore, variations in the amount of

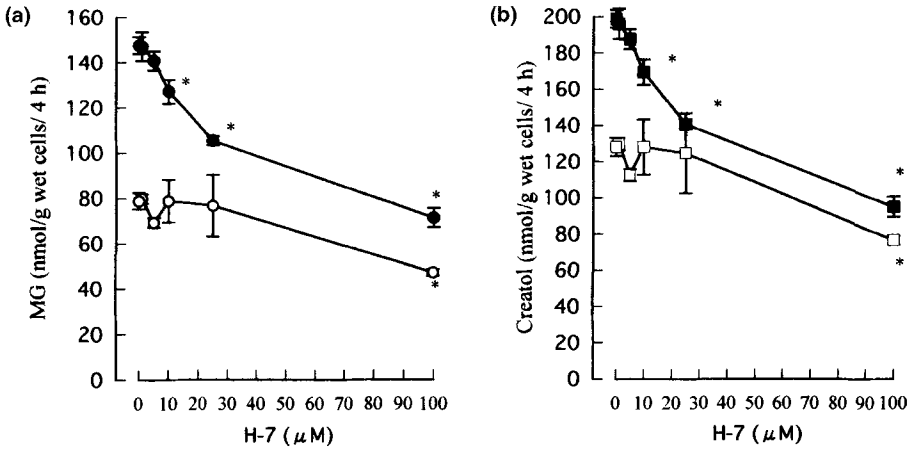

FIGURE 3 Dose dependent inhibition by H-7 of MG or creatol synthesis in isolated rat hepatocytes. Isolated hepatocytes (0.06 g wet cells) were incubated for 4 h as described in Materials and Methods. MG synthesis is shown in (a) and creatol synthesis is shown in (b). MG with or without PAN are indicated by ● and ○, respectively, ■ and □ indicate creatol with or without PAN. Each point represents the mean of four incubations. Bars indicate the standard error. * represents p less than 0.05 vs. the control value.

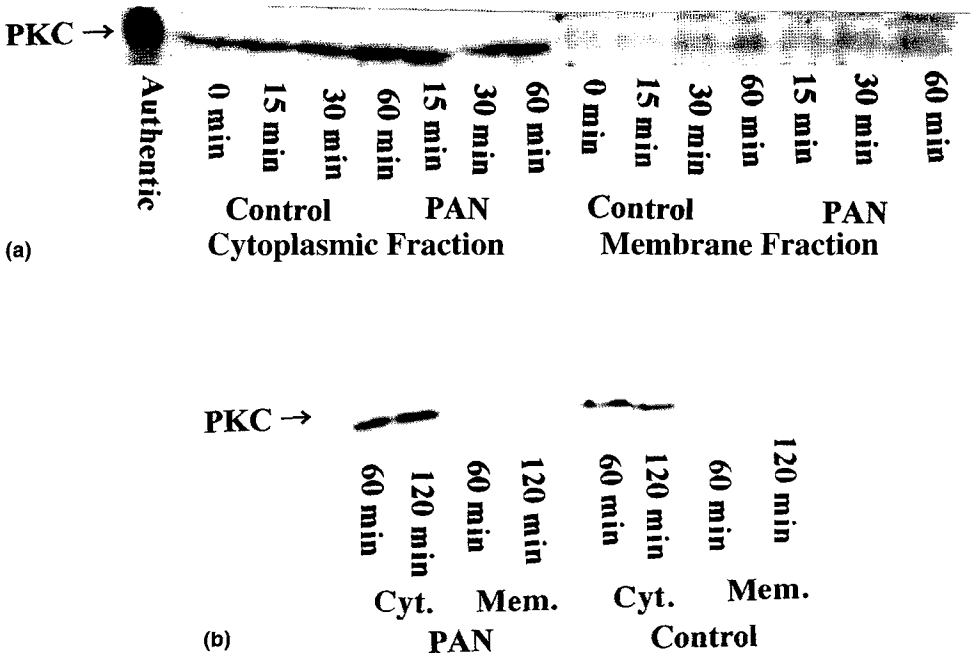

FIGURE 4 Western blotting of PKC from control and PAN-treated isolated rat hepatocytes. PKCs were detected by antibodies against the PKC α, β and γ isozymes in the membrane and cytoplasmic fraction as described in Materials and Methods. PKCs from isolated hepatocytes incubated 0, 15, 30 and 60 min are shown in (a). PKCs from isolated hepatocytes incubated with or without PAN for 60 and 120 min and detected in a second gel are shown in (b). Cyt.: Cytoplasmic fraction, Mem.: Membrane fraction.

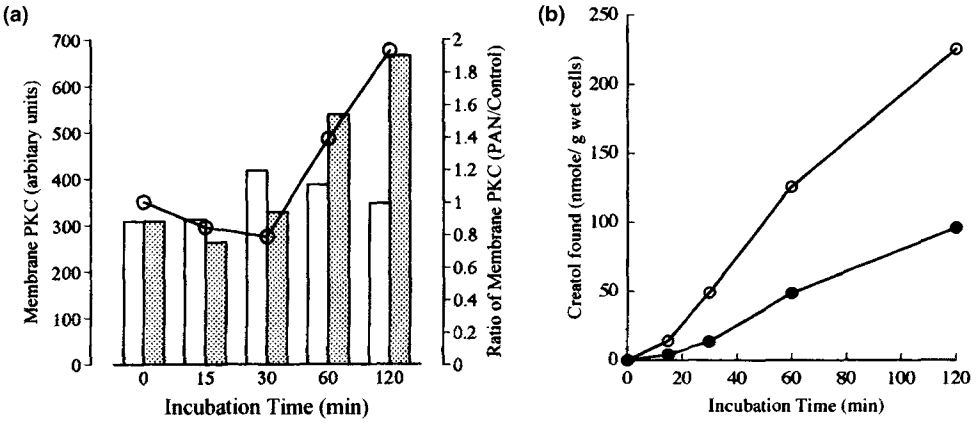

FIGURE 5 PKC activation by PAN in isolated rat hepatocytes. Isolated rat hepatocytes (1.5 g wet cells) were incubated in 35 ml Krebs–Henseleit buffer as described in Materials and Methods. PKCs in the membrane fraction without PAN are expressed as open columns, with PAN are expressed by closed columns. The PKC ratio in the membrane with PAN to that without PAN are expressed as open circles in Figure 5(a). Creatol synthesis, determined in the same sample, is shown in (b). ○ indicates with PAN, ● indicates without PAN.

PKC per unit of protein in the cytosol with or without PAN of less than 30% were omitted.

Creatol was determined in the same samples as that used for testing PKC activation. Creatol levels in the presence of PAN at 30, 60 and 120 min incubation increased 3.2, 3.0 and 2.3 times that seen in the absence of PAN (Figure 5(b)). The coincidental activation of PKC and increase in creatol synthesis suggest that PKC is involved in the increase in hydroxyl radical generation induced by PAN.

DISCUSSION

This is the first report that PAN activates PKC resulting in increased hydroxyl radical generation. These data also indicate that the activation of conventional type PKC is involved in hydroxyl radical generation in tissue cells as well as in inflammatory cells.

Nephrosis induced by PAN has been extensively investigated because PAN induces heavy proteinuria within one week without infiltration of inflammatory cells and its features resemble lipoid nephrosis as seen mostly in children. An increased reactive oxygen generation by PAN has been reported.[3–7,26] We have also reported that PAN increased hydroperoxide synthesis in isolated rat glomeruli *in vitro* as well as in isolated glomeruli obtained from PAN-induced nephrotic rat kidneys as shown by the imaging of fluorescence from peroxides and dichlorofluorescine.[27]

However, the mechanism for the increased reactive oxygen induced by PAN has not been determined. The methodological difficulty in the quantitative assay for reactive oxygen in non-inflammatory cells prevented investigation of this point. We overcome this problem using MG and creatol for assay of the hydroxyl radical.[5–10,28] MG is a guanidino compound and its synthesis is increased in patients with renal failure. Marked increases in MG were observed when the patients develop uremic symptoms. MG has a variety of actions such as inhibition of gamma-amino butyric acid (GABA) receptors,[29] nitric oxide synthetase[30] and ATPase,[31] and causes such uremic symptoms as convulsions, shortening of life span of uremic rats and hypertension.[32–34] Recently, serum levels of N^{ϵ}-(carboxymethyl)-lysine, an amyloid precursor produced by ROS, correlated with MG levels in patients under hemodialysis.[35]

Nishizuka reported that PKC plays an important role in cell function.[14] In leukocytes, activation of PKC resulted in the generation of reactive oxygen.[13] Recently, many isoforms have been reported.[36] Among them, α, β and γ isoforms are activated by both diacylglycerol and Ca^{2+} are called conventional type PKC. Conventional type PKC play an important role in the activation of leukocytes for the generation of reactive oxygen.[37]

In this paper, we show that H-7 and Ro31-8425, PKC inhibitors suppressed MG and creatol synthesis in concentrations as low as 10 μM. H-7 competes with ATP and the K_i value of H-7 for PKC is 6 μM.[38] H-7 acts as a PKC inhibitor in many cells including hepatocytes.[39–42] Ro31-8425, a potent and specific PKC inhibitor, also acts in hepatocytes.[41,42] H1004, used as an inactive control for H-7, did not affect MG synthesis at 10 μM and, in fact, increased MG synthesis at 100 μM. A reduced inhibition of MG and creatol synthesis by H-7 in the absence of PAN suggests that the process of preparation of isolated rat hepatocytes itself activates PKC to a certain extent.

To demonstrate the activation of PKC in cells, translocation or down regulation of PKC after activation has been reported as a useful indicator.[14,25,26] In this report, we demonstrate that PAN activates conventional type PKC by showing the rapid translocation of PKC from the cytoplasmic to the membrane fraction and the increased hydroxyl radical generation coincident with this PKC activation. However, we did not observe this down regulation of PKC within 2 h in this experiment.

The mechanism of increased hydroxyl radical generation by PKC activation in hepatocytes

should be different from that seen in inflammatory cells because hepatocytes have no NADPH-oxidase system such as that in the membrane that generates ROS in inflammatory cells by stimulation of PKC. Activation of PKC in liver causes cholestasis,[43] stimulation of apical exocytosis,[44] mitogen-activated protein kinase activation[41] and phospholipase D activation.[42] Among these effects, activation of phospholipases could lead to an increase in hydroxyl radical generation through arachidonic acid.[41,42,45] However, recently, the NADPH-oxidase system capable of generating ROS through stimulation of PKC was reported in smooth muscle cells.[46,47] NADPH-oxidase activity in cell membranes in the hepatocyte is limited to the bile-canalicular plasma membrane[48] and whether PKC activates NADPH-oxidase in the bile canalicular cell membrane is still unclear.

Acknowledgments

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