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Synthesis of Creatol, a Hydroxyl Radical Adduct of Creatinine and its Increase by Puromycin Aminonucleoside in Isolated Rat Hepatocytes

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Creatol is a hydroxyl radical adduct of creatinine and the precursor of methylguanidine (MG), a uremic toxin. We investigate the synthesis of creatol and MG from creatinine and the effect of substances that affect the hydroxyl radical in isolated rat hepatocytes. In the presence of increasing concentrations of creatinine, rising level of creatol were found after 2 h incubation in Krebs–Henseleit bicarbonate buffer. However, further increase of creatol was not observed after 4 and 6 h incubations. On the other hand, MG after 2 h incubation achieved a level of about 50% that of creatol and increased depending on both the creatinine concentration and the incubation period. DMSO, a hydroxyl radical scavenger decreased the generation of creatol and MG by about 50% at 2.5 mM and the inhibition depended on DMSO concentration. Puromycin aminonucleoside (PAN) increased both by about 170%. These findings demonstrated that hepatocytes synthesize creatol prior to MG and are inhibited by a hydroxyl radical scavenger. They also show that PAN increased hydroxyl radical generation in tissue cells.

Keywords: Creatol, methylguanidine, hydroxyl radical, puromycin aminonucleoside

INTRODUCTION

Recently, free radicals have been implicated in a variety of diseases and injuries.[1] Among the several species of free radicals, the hydroxyl radical has been recognized as the most harmful.[2] A direct assay, however, for the hydroxyl radical *in vivo* is difficult because of its short life span.

We have found that methylguanidine (MG), a uremic toxin,[3] is formed from the reaction of creatinine with the hydroxyl radical. This reaction has been confirmed *in vitro*,[4] and also in

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the presence of activated human leukocytes.[5] In addition, it has been shown that the ratio of MG to creatinine increases in the urine of subjects exposed to hyperbaric oxygen chamber.[6] We also reported that puromycin aminonucleoside (PAN), a substance used to induce heavy proteinuria, increases MG synthesis from creatinine both in isolated rat hepatocyte[7] and in rats.[8]

Recently, the intermediate that is the direct product of creatinine with the hydroxyl radicals is identified as creatol (CTL) and confirmed as a precursor of MG.[9]

In this paper, we investigated the synthesis of CTL and MG from creatinine under various conditions and demonstrate in isolated rat hepatocytes the value of MG as a marker for the hydroxyl radical. We also investigated under similar conditions the effect of PAN, which generates both heavy proteinuria and the hydroxyl radical, on the synthesis of CTL and MG.

MATERIALS AND METHODS

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[10] as described previously.[11] We calculated that 9.8×10^7 cells correspond to 1 g wet liver.[12]

Incubation of Cells

Cells were incubated in 6 ml of Krebs-Henseleit bicarbonate buffer containing 3% bovine serum albumin, 10 mM sodium lactate, concentrations of creatinine as indicated and other substances as noted. 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. After sonication, the supernatant of cells and medium was obtained by centrifugation at 1,700*g* for 15 min at 0°C, and 0.2 ml of the extract was used for MG or CTL measurements.

Assay of MG and CTL

MG was determined by high-performance liquid chromatographic analysis using 9,10-phenanthrenequinone for the postlabeling method as described previously.[13] CTL was determined by HPLC after conversion from CTL to MG according to the method reported by Nakamura *et al.*[14] Dimethylformamide for fluorometrical use and 9,10-phenanthrenequinone were purchased from Wako, Japan. PAN was purchased from Sigma Chemical, St. Louis.

RESULTS

Syntheses of MG and CTL in Isolated Rat Hepatocytes

The time course for the appearance of CTL, MG and MG + CTL in the supernatant are shown separately according to the creatinine concentration in Figure 1(a)–(c). MG increased almost in proportion to the incubation period. However, CTL reached its maximum level after 2 or 4 h incubation and either remained steady or decreased at 6 h. After 2 h of incubation, CTL levels were about 1.5–2.0 times higher than those of MG at any concentration of creatinine. These results suggest that CTL is formed prior to MG. After 4 h incubation, CTL achieved levels similar to those of MG. The combined level of MG + CTL increased relative to the incubation period.

MG increased in proportion to the creatinine concentration and the incubation period (Figure 1(d)). However, at 16.6 mM creatinine, slightly less MG was formed after 2 h incubation than was expected. This suggests that conversion

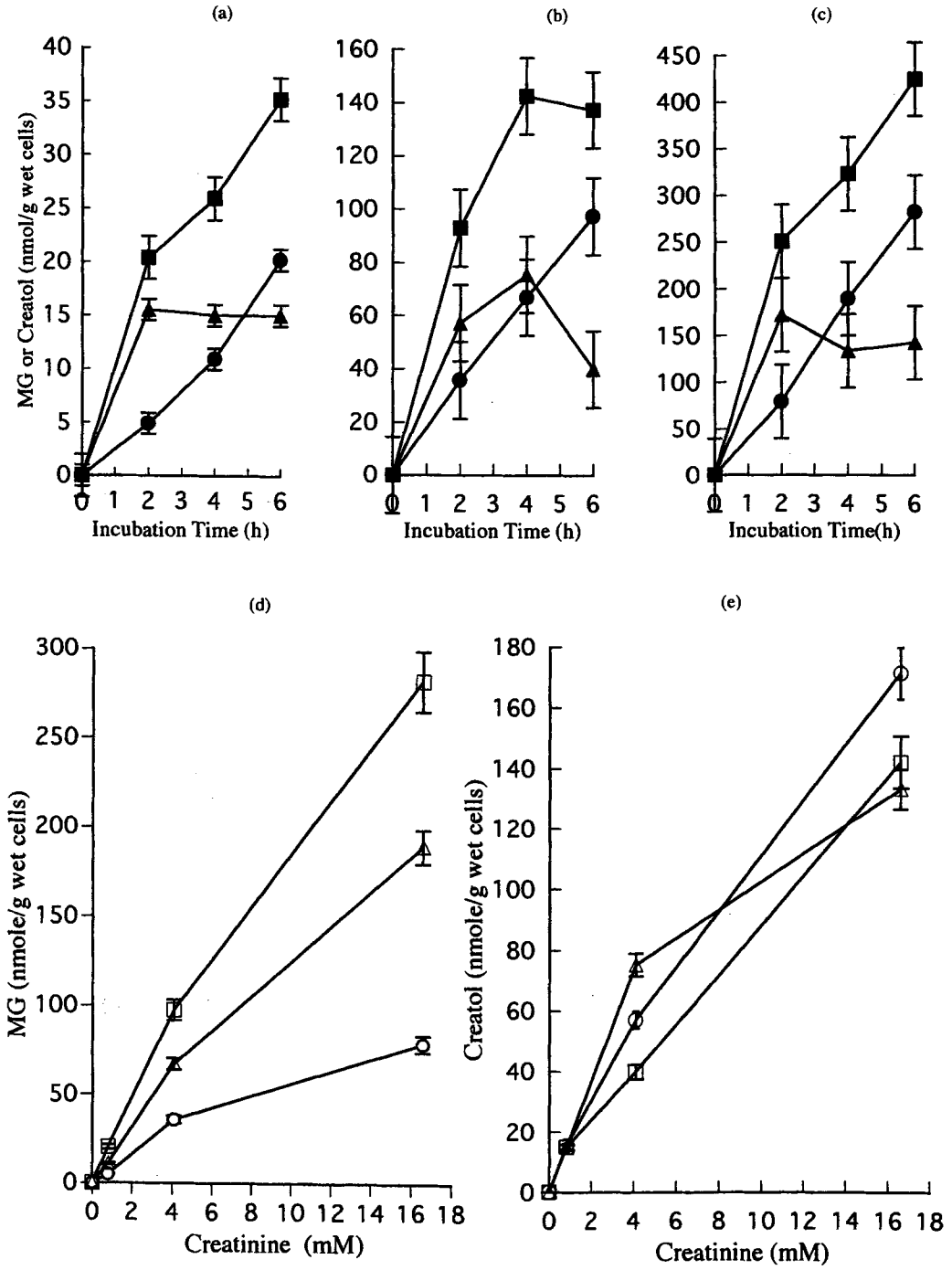

FIGURE 1 Synthesis of CTL and MG in isolated rat hepatocytes. Cells (0.10 g wet weight) were incubated with the indicated concentrations of creatinine as described in Materials and Methods for 2, 4 and 6 h. The amount of MG (●), CTL (▲) and MG+CTL (■) found in the supernatants in the presence of 0.8, 4.1 and 16.6 mM creatinine are shown in (a), (b), and (c), respectively. MG and CTL concentrations found in the presence of various levels of creatinine after 2 h (○), 4 h (△) and 6 h (□) are shown in (d) and (e) respectively. Each point represents the mean of duplicate incubations. Bars indicate the range of each determination.

from CTL to MG may be limited after 2 h incubation at creatinine concentrations as high as 16.6 mM.

CTL in the incubation medium increased in proportion to the creatinine concentration as shown in Figure 1(e).

Effect of DMSO on CTL and MG Synthesis

We have reported that DMSO, a hydroxyl radical scavenger inhibited MG synthesis in isolated rat hepatocytes.[6] DMSO at the concentration from 0.5 to 37.5 mM inhibited CTL synthesis in the presence of 16.6 mM creatinine depending on its concentration at 2 and 4 h as shown in Table I. At the same time, DMSO also inhibited MG synthesis at the same extent of the inhibition of CTL synthesis at 2 and 4 h, respectively.

We propose that DMSO inhibits MG synthesis because of its activity as a scavenger of the hydroxyl radical. However, the possibility that DMSO inhibits the conversion from CTL to MG still exists. These findings confirm that DMSO inhibits CTL synthesis in isolated rat hepatocytes.

Basically, the mode of the inhibition is competitive. However, the inhibition rate of around 30% at 0.5 mM DMSO is much higher than the inhibition rate calculated from the data at 7.5 mM DMSO. Therefore, DMSO at the low concentration of 0.5 mM may diminish the hydroxyl radical generation by the cells as well and scavenge the hydroxyl radical as well.

Effect of PAN on CTL and MG Synthesis

MG and CTL in the supernatant after incubation with or without PAN are shown in Figure 2. PAN increases MG synthesis by about 2-fold throughout all periods of incubation. PAN also increased

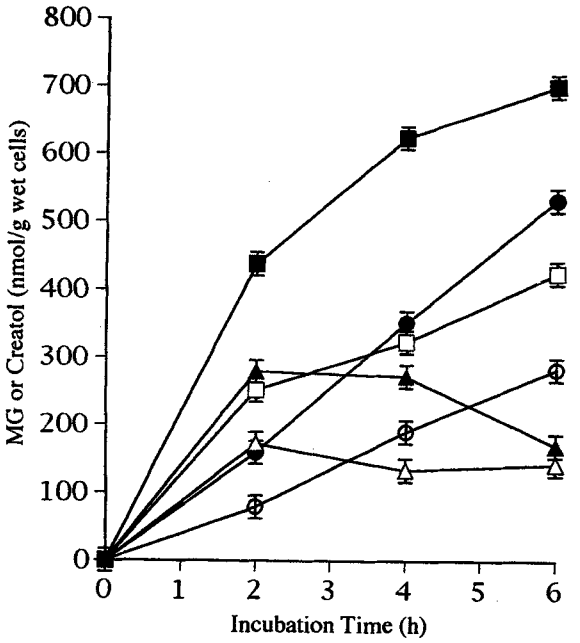

FIGURE 2 Increased synthesis of MG and CTL by PAN in isolated rat hepatocytes. Cells (0.10 g wet weight) were incubated with 16.6 mM creatinine with or without 1.9 mM PAN as described in Materials and Methods for 2, 4 and 6 h. The symbols MG (○), CTL (△) and MG + CTL (□) refer to those without PAN and MG (●), CTL (▲), and MG + CTL (■) are those with PAN. Each point represents the mean of duplicate incubations. Bars indicate the range of each determination.

TABLE I Effect of DMSO on CTL and MG synthesis in isolated rat hepatocytes. Cells (0. 16 g wet weight) were incubated with 16.6 mM creatinine and indicated concentration of DMSO as described in Materials and Methods. Values (nmol/g wet cells) are expressed as the mean ± SEM (Controls: $n = 5$, Others: $n = 2$). Data were subjected to ANOVA with pairwise comparison by the Bonferroni method

DMSO (mM)	nmol/g wet cells (Inhibition rate, %)					
	2 h MG	2 h CTL	2 h MG + CTL	4 h MG	4 h CTL	4 h MG + CTL
0	26 ± 1.0 (0)	282 ± 19 (0)	307 ± 18 (0)	83 ± 1.6* (0)	240 ± 16* (0)	324 ± 17* (0)
0.5	19 ± 1.1* (26)	166 ± 2.2* (33)	208 ± 4.8* (32)	60 ± 0* (27)	164 ± 4.5* (32)	224 ± 6.3* (31)
2.5	15 ± 0* (43)	141 ± 0* (50)	156 ± 0* (49)	43 ± 1.6* (48)	141 ± 0* (41)	184 ± 2.3* (43)
7.5	9 ± 1.0* (65)	90 ± 2.7* (68)	99 ± 5.2* (68)	27 ± 0.6* (67)	95 ± 3.1* (60)	122 ± 3.5* (62)
37.5	6 ± 0* (77)	68 ± 2.7* (76)	74 ± 3.8* (76)	19 ± 0.6* (77)	66 ± 0.9* (73)	85 ± 0.3* (74)

*$P < 0.05$ vs. the control value.

CTL by about 2 times after 2 and 4 h incubation. However, after 6 h incubation the increase of CTL by PAN was not observed suggesting that the PAN-induced increase in hydroxyl radical generation dose not persist after 4 h.

DISCUSSION

In this study, we showed that isolated rat hepatocytes can form CTL. Also under a variety of conditions, MG increased linearly depending on both the concentration of creatinine and the incubation time. However, the CTL formed did not depend on the incubation period. This suggests that the CTL was converted to MG. Since the amount of CTL found in the homogenate is dependent upon both its synthesis and degradation rates, the determination of the sum MG + CTL is a better index of the rate of generation of the hydroxyl radical than estimation of MG alone. However, MG synthesis after a 4 h incubation at 16.6 mM creatinine concentration yielded a quantitative estimate of the amount of creatinine oxidized which correlated well with the data obtained from estimates of MG + CTL. These findings confirm our previous reports in which we estimated hydroxyl radical generation in isolated rat hepatocytes.[7,15]

Conversion of CTL to creaton A is carried out by both enzymatic[16] and non-enzymatic reactions.[17] The enzymes that form creaton A from creatol were purified from rat liver[18] and kidney.[19,20] Non-enzymatic conversion of CTL to creaton A was carried out by both an oxidative and non-oxidative reaction. However, the rate of chemical reaction is much lower than that of the enzymatic conversion.[17] Moreover, the enzyme that converts CTL to MG is not the specific for CTL and may actually have a high Km for CTL. The conversion from CTL to MG is in proportion to the CTL concentration which also suggests a high Km for CTL.

The CTL concentration at 4 h incubation was usually lower than MG and also lower than the CTL concentration of 2 h incubation. However, sometime the CTL concentration is higher than that of MG and also higher than the CTL concentration of 2 h incubation in similar experimental conditions (data not shown). The limited conversion from CTL to MG accounts for this discrepancy. The reason for this limited conversion is not clear. However, the over-digestion of the liver by collagenase may explain this limited conversion.

The significance of CTL lies in the fact that it is a direct product of the reaction of creatinine with the hydroxyl radical. The demonstration of the increased CTL synthesis is an important index of increased hydroxyl radical generation.

In this paper, we show that PAN increased CTL production suggesting that it, too, increases hydroxyl radical generation. PAN induces heavy proteinuria and the increase in reactive oxygen may well be the cause of the heavy proteinuria as suggested by experiments using scavengers of reactive oxygen.[21] In this paper, we demonstrate that PAN increases hydroxyl radical generation in tissue cells directly.

Creatinine is an endogenous substance of low toxicity that is widely distributed. Therefore, determination of MG and CTL is a useful method to assay for hydroxyl radicals *in vivo* as well as *in vitro*.

Acknowledgments

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