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SHORT COMMUNICATIONS

Ketogenesis in isolated rat hepatocytes
Effect of oleate and chlorpropamide on ketogenesis from endogenous lipids

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The hypoglycemic drug chlorpropamide (5 mM) has been shown to inhibit ketogenesis from endogenous lipids by 80 per cent in livers of fasted rats [1] and by 40 per cent in livers of alloxan-diabetic rats perfused without fatty acid substrate [2], but no effect of the drug on ketogenesis could be observed when fasted livers were perfused with either 1 mM oleate or with 5 mM octanoate [2]. These results suggested that chlorpropamide could interfere with hepatic triglyceride breakdown but not with fatty acid oxidation. However, since the effect was most pronounced in those situations where fatty acid oxidation was relatively low (e.g. fasted livers perfused without fatty acids), the possibility remained that chlorpropamide inhibited ketogenesis at some point in a competitive manner. This possibility has now been further examined using isolated rat hepatocytes and a wider range of substrate and drug concentrations. The use of [1-^{14}C]oleate as substrate and measurements of the specific radioactivity of the ketone bodies formed allowed for the discrimination between ketone bodies derived from the radioactive exogenous substrate and from unlabeled endogenous lipids (mainly triglycerides), so that the effect of chlorpropamide on both sources of ketogenesis could be examined.

MATERIALS AND METHODS

Hepatocytes were prepared from livers of fasted rats by the method of Zahlten *et al.* [3]. 5×10^6 cells were incubated in duplicate in siliconized glass scintillation vials in a final vol. of 2 ml of Krebs–Henseleit bicarbonate buffer, containing 2.3 g per 100 ml of defatted albumin, 0.1–1 μmole per ml of [1-^{14}C]oleate and 1–5 μmoles per ml of chlorpropamide. 1 μmole of L-carnitine per ml of incubation mixture was routinely added since preliminary experiments had shown that with some cell preparations carnitine stimulated ketogenesis, possibly as a consequence of a variable washout of carnitine reported to occur during isolation of hepatocytes[4]. The vials were gassed with O_2:CO_2 (95:5, v/v) and incubated with shaking at 37° for 30 or 60 min. More than 80 per cent of the cells excluded trypan blue at the end of a 60 min incubation. The reactions were stopped with perchloric acid and β-hydroxybutyrate [5] and acetoacetate [6] were determined enzymatically in the neutralized supernatant. Ketogenesis refers to the production of β-hydroxybutyrate plus acetoacetate. After conversion of β-hydroxybutyrate to acetoacetate the radioactivity associated with the carbonyl and carboxyl group of acetoacetate was measured according to McGarry *et al.* [7]. Appropriate corrections for blanks and recoveries were always performed. The known sp. act. of the substrate allowed for the calculation of the amount of the ketone bodies derived from the radioactive substrate (exogenous ketogenesis). The difference between the total and the radioactive ketones from exogenous oleate gave the amount derived from hepatic lipid stores (endogenous ketogenesis). The adequacy of the methodology is well established [7, 8].

RESULTS

In a first series of experiments, the effect of chlorpropamide on endogenous ketogenesis of isolated hepatocytes incubated in a fatty acid free medium was examined. Over a 60 min period endogenous ketogenesis proceeded at the rate of 17.56 ± 1.35 μmoles ketone bodies $\times$ 60 min^{-1} $\times$ 10^8 cells^{-1} (mean ± S.E.M.) and was reduced to 4.19 ± 0.57 μmoles $\times$ 60 min^{-1} $\times$ 10^8 cells^{-1} in the presence of 2.5 mM chlorpropamide ($n = 3$, $P < 0.05$).

The effects of chlorpropamide on ketone body production by liver cells in the presence of 0.1–1.0 mM [1-^{14}C]-oleate are shown in Fig. 1. During the first half hr of incubation (Fig. 1A) 1 mM chlorpropamide significantly inhibited total ketogenesis in the presence of 0.1, 0.3 and 0.5 mM oleate by 18, 13 and 9 per cent respectively. The inhibition was always the result of a significantly reduced contribution of endogenous lipids. The effect of chlorpropamide was dose-dependent. Higher drug concentrations tended to slightly inhibit exogenous ketogenesis but the effect was significant in only two cases and at the highest drug concentration. At 1.0 mM oleate concentration, a significant inhibition of endogenous and total ketogenesis could only be demonstrated in the presence of 5 mM chlorpropamide. Essentially the same pattern of inhibition was observed during the second half hr of incubation (Fig. 1B). Surprisingly, at all substrate concentrations, total ketogenesis during the second half hr proceeded almost at the same rate as during the initial half hr, despite a decrease of the substrate concentration in the medium. This may be explained by the results of a series of control experiments depicted in Fig. 2. Total ketogenesis increased linearly with substrate concentration. However, with increasing substrate concentrations the contribution of the exogenous fatty acid leveled off, while ketogenesis from endogenous lipids increased. This phenomenon was most apparent during the second half hr (Fig. 2B) where at 1 mM oleate concentration, endogenous ketogenesis increased to almost three times the rate observed in the absence of substrate, accounting for 67 per cent of the total ketogenesis.

DISCUSSION

In addition to its inhibiting effect on ketogenesis by livers perfused without substrate, chlorpropamide has now been shown to retain its inhibiting effect on endogenous ketogenesis in liver cells incubated in the presence of exogenous fatty acids. The marked reduction of endogenous ketogenesis caused by chlorpropamide sharply contrasts with its small effect on exogenous ketogenesis, which reached the level of significance at the highest drug concentration only. Therefore, the present observations corroborate the earlier suggestion by us [1, 2] and others [9] that sulfonylureas primarily affect hepatic ketogenesis through interference with hepatic triglyceride breakdown. Noteworthy is the fact that the inhibitory effect of chlorpropamide on endogenous ketogenesis strongly diminishes when

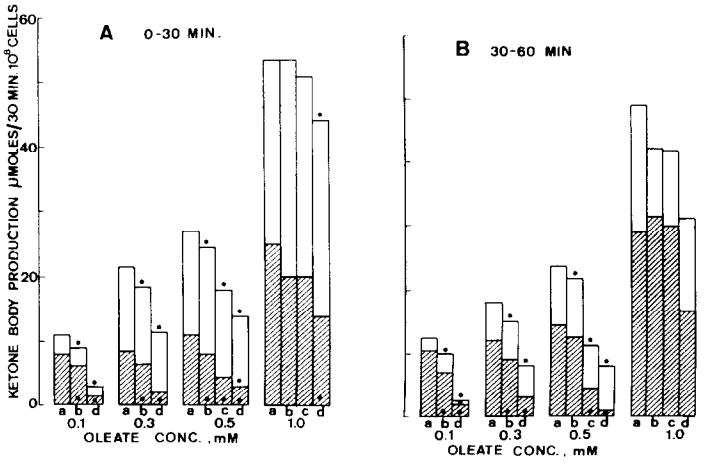

Fig. 1. Liver cells of fasted rats were incubated for 60 min as described in Methods. Fig. 1A presents the results obtained during the first half hr, Fig. 1B those of the second half hr of incubation. Each bar represents the mean of at least 4 different liver cell preparations. Total ketogenesis is represented by the total height of a column; it has been subdivided into its endogenous (shaded area) and exogenous (white area) components as described in Methods. A star on top of a column indicates a statistically significant difference (at least $P < 0.05$) versus the corresponding control total ketogenesis; within either the shaded or the white area, it refers to the corresponding area of the control. Significance of difference between means was established by Student's t-test for paired data. a = control experiments, b = + 1 mM chlorpropamide, c = + 2.5 mM chlorpropamide, d = + 5 mM chlorpropamide.

the cells are incubated in the presence of 1 mM oleic acid. This decrease in inhibitory action of chlorpropamide is accompanied by a strong stimulation of endogenous ketogenesis by oleate (see further), and it is therefore likely that both phenomena are related.

The moderate inhibition of endogenous ketogenesis observed in the presence of therapeutic concentrations of chlorpropamide (1 mM or less) precludes any significant effect of the drug on ketogenesis in diabetic patients. However, an interference of the drug with triglyceride breakdown could affect hepatic triglyceride content and secretion which plays a central role in blood lipid homeostasis. Studies on the effect of chlorpropamide on hepatic triglyceride metabolism performed in this laboratory will be published in extenso elsewhere [10].

The data of Fig. 2 are at variance with those of Ontko [11] who reported a decrease of endogenous ketogenesis with increasing substrate concentration. Whether this discrepancy is due to differences in methodology is not clear. Since up to 30 per cent of the substrate taken up by liver cells of fasted rats can be esterified [11] the stimulatory effect of oleate on endogenous ketogenesis could be interpreted as the result of an increase in hepatic triglyceride content and thus an increase in endogenous ketogenesis. This process would be an energy wasting futile cycle that could explain in part the very low ADP/O ratio determined by us for the oxidation of 1 mM oleate by the perfused livers of fasted rats [12]. However, esterification of incoming oleate resulting in a mobilization of preexisting triglycerides is probably not the only mechanism responsible for the observed increase in endogenous ketogenesis. Indeed, when liver cells were incubated in the presence of 1 mM octanoate total ketogenesis was 60.62 ± 1.87 μmoles × 30 min^{-1} × 10^8 cells^{-1} and endogenous keto-

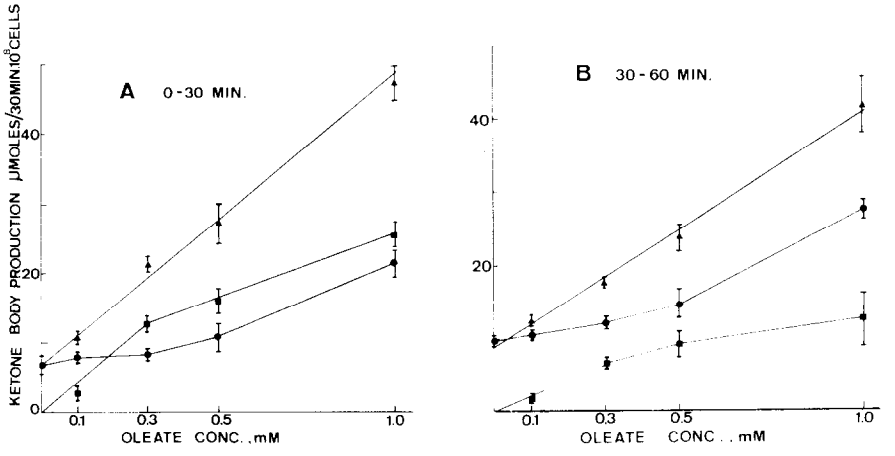

Fig. 2. Incubation conditions and the meaning of total, endogenous and exogenous ketogenesis have been explained in Methods. Each point represents the mean ± S.E.M. of at least 5 different liver cell preparations. —▲— total ketogenesis, —■— exogenous ketogenesis, —●— endogenous ketogenesis.

genesis was as high (22.64 ± 2.93 μmoles × 30 min^{-1} × 10^8 cells^{-1}, $n = 4$) as with 1 mM oleate. Since octanoate is not esterified by the liver [13] it appears that not only esterification but rather the overall metabolic state of liver cells rapidly utilising free fatty acids is responsible for the observed labilization of endogenous triglycerides. The details of the mechanism(s) involved remain to be elucidated. They could provide valuable information pertaining to the situation in the perfused liver of diabetic rats which produces ketone bodies at a high rate, regardless whether fatty acids are present in the perfusion medium or not [14]. It is possible that in this situation, as in the experiments reported here, the continuous increased supply of fatty acids to the liver *in vivo* would not only sustain the high concentration of hepatic triglycerides but also accelerate their turnover.

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Effects of local anesthetics and cholesterol on the ($Na^+ + K^+$)-dependent ATPase

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The ($Na^+ + K^+$)-dependent ATPase represents the biochemical basis for transporting Na^+ and K^+, the sodium pump, and is present *in vivo* in the plasma membrane [1]. Among the consequences of this localization is the sensitivity of the enzyme to membrane structure. Early studies showed that alternations in membrane lipids (such as enzymatic digestion or solvent extraction) drastically decreased activity, and that activity could be restored by replacing lipids [2–5]. Nevertheless, disagreements about the relative importance of various lipid classes have been difficult to resolve. For example, Wheeler and Whittam [4] obtained optimal restoration of ATPase activity only when phosphatidyl serine was added, and Noguchi and Freed [5] found that the presence of cholesterol was crucial, whereas Hilden and Hokin [6] were able to restore activity when the only lipid present was phosphatidyl choline.

The experiments presented here are concerned with another aspect of enzyme–lipid interactions, the effects on enzyme activity of agents known to interact with the lipid bilayer: cholesterol and two local anesthetics, procaine and benzyl alcohol (one largely ionized at the pH used and the other unionized). These agents were studied in terms of their effects on cation activation both of the ($Na^+ + K^+$)-dependent ATPase and of the related K^+-dependent phosphatase reaction, which appears to reflect the terminal hydrolytic steps of the overall ATPase reaction [1]. The data demonstrate differences between the three agents in effects on several kinetic parameters, as well as generally greater effects at an incubation temperature below the lipid phase transition temperature of the membrane.

The enzyme preparation used was obtained from rat brain microsomes after treatment with deoxycholate and NaI, as described previously [7]. For addition of cholesterol, a suspension of 0.2 mM cholesterol in 60 mM histidine-HCl/Tris (pH 7.8) was sonicated to translucence, and then mixed with an equal volume of the enzyme preparation 10 min before initiating the assay. The final concentration of cholesterol in the incubation medium was 0.01 mM. Controls without cholesterol were run concurrently.

($Na^+ + K^+$)-dependent ATPase activity was measured in terms of the production of P_i, as previously described [7]. The standard medium contained 30 mM histidine-HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, 10 mM KCl, and the enzyme preparation (0.1 mg protein/ml). Activity in the absence of Na^+ and K^+ was measured concurrently; such activity averaged only a few per cent of the ($Na^+ + K^+$)-dependent ATPase activity [7], and was subtracted from the total activity in the presence of Na^+ and K^+ to give the ($Na^+ + K^+$)-dependent activity. Because of variations in the absolute activity of different enzyme preparations, enzyme velocities are expressed relative to the ($Na^+ + K^+$)-dependent ATPase activity of the concurrent control incubation in the standard medium, defined as 1.0. K^+-dependent phos-