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Short communication

Effects of 5-iodotubercidin on hepatic fatty acid metabolism mediated by the inhibition of acetyl-CoA carboxylase

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Abstract

Diverse mechanisms of action have been proposed for 5-iodotubercidin, although it is widely used as an adenosine kinase inhibitor that consequently interferes with the metabolism of adenosine and adenine nucleotides. Incubation of rat hepatocytes with iodotubercidin produced important effects on lipid metabolism. (i) Both acetyl-CoA carboxylase and fatty acid synthesis *de novo* were inhibited in parallel by iodotubercidin, with no change in the activity of fatty acid synthase. The inhibition of both activities showed a comparable dependence on iodotubercidin concentration and was accompanied by a similar decrease (about 60%) in the intracellular malonyl-CoA concentration. (ii) Iodotubercidin stimulated palmitate oxidation, although octanoate oxidation was unaffected. However, this effect can be attributed to the decrease of malonyl-CoA concentration and the concomitant relief of the inhibition of carnitine palmitoyltransferase I, because the activity of this enzyme was found unaltered when determined in cells permeabilized with digitonin. (iii) Iodotubercidin also inhibited cholesterol synthesis *de novo*. Results, thus, indicate that iodotubercidin increases fatty acid oxidation activity of the liver at the expense of lipogenesis, and we suggest that these effects on fatty acid metabolism are mediated by the inhibition of acetyl-CoA carboxylase, probably due to a more than twice increase in the AMP/ATP ratio and the concomitant stimulation of the AMP-activated protein kinase.

Keywords: Iodotubercidin; Fatty acid metabolism; Acetyl-CoA carboxylase; Carnitine palmitoyltransferase I; AMP; Hepatocyte

1. Introduction

Adenosine, acting through four subtypes of G protein-coupled receptors, has many biological functions that afford the bases of a variety of therapeutically useful pharmacologies [1]. 5-Iodotubercidin (Itu) is a potent inhibitor of adenosine kinase [2], and therefore, it has been widely used to study the effects of adenosine, as well as to determine the role of adenosine kinase in the metabolism of adenosine in different cell types [3]. However, much less is known about the effects of iodotubercidin on intermediary metabolism. Thus, it has been found that, independently of the addition of adenosine, Itu stimulates glycogen synthesis in hepatocytes, in parallel to an increase in glycogen synthase *a* and a decrease in phosphorylase *a* concentrations, but these effects could not be explained by the catabolism of ATP or the inhibition of adenosine kinase [4]. A possible mechanism has been proposed for these effects of Itu, suggesting that this compound acts as a general inhibitor of protein kinases [5], although this cannot be the mechanism responsible of other effects, such as the inhibition of glycolysis in hepatocytes [6]. In fact some other related mechanisms of action have been proposed for Itu, namely the inhibition of AMP-activated protein kinase (AMPK) [7,8] and more recently, the inhibition of extracellular-signal regulated kinase ERK2 [9].

In contrast with the important documentation about the effects on glycogen metabolism, to the best of our knowledge, Itu has never been used in studies on lipid metabolism. However, we hypothesized that this could be a good model to study the actions of iodotubercidin, because this agent may alter intracellular levels of adenine nucleotides, with a concomitant change in the activity of the AMPK. This protein kinase is currently considered as a ‘‘master switch’’ that plays a critical role in the regulation of cellular processes which are controlled by the energy state [10–12]. Thus, AMPK plays a central role in the regulation

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Abbreviations: Itu, 5-iodotubercidin; ACC, acetyl-CoA carboxylase; CPT-I, carnitine palmitoyltransferase I; FAS, fatty acid synthase; AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide.

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of lipid metabolism by inhibiting key biosynthetic enzymes, such as acetyl-CoA carboxylase (ACC) in fatty acid synthesis and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in cholesterol synthesis [13]. Modulation of ACC is also essential for the control of carnitine palmitoyltransferase I (CPT-I), that is a key regulatory site of ketogenesis in hepatocytes [14,15]. CPT-I is inhibited by malonyl-CoA, the product of the reaction catalyzed by ACC, so that coordinate control of synthesis and oxidation of fatty acids is achieved [14,15]. Thus, in the present study, we examined in detail the effects of 5-iodotubercidin on the different fatty acid-metabolizing pathways and on the activities of these two key enzymes, ACC and CPT-I.

2. Materials and methods

2.1. Materials

Radiochemicals were all supplied by Amersham Pharmacia Biotech. Collagenase, digitonin, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), propentofylline and 3,7-dimethyl-1-propargylxanthine (DMPX) were purchased from Sigma Chemical Co. 5-Iodotubercidin (4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine) was from RBI. Tetradecylglycidic acid (TDGA) was a kind gift from J.M. Lowenstein, Brandeis University (Waltham, MA, USA).

2.2. Isolation and incubation of hepatocytes

Hepatocytes were isolated from male Wistar rats (250–300 g) by the collagenase perfusion method [16] and incubated in Krebs–Henseleit bicarbonate buffer supplemented with 10 mM glucose and 1% (w/v) defatted and dialyzed bovine serum albumin. Cells (2 mL; 4–6 mg of cellular protein/mL) were incubated with additions at 37° for 30 min, with constant shaking under an atmosphere of O_2/CO_2 (19:1). For the determination of rates of fatty acid synthesis *de novo*, reactions were started by the addition of 3 mM [1-^{14}C]acetate to hepatocyte incubations. After 30 min, reactions were stopped and total fatty acids were extracted [16]. Rates of fatty acid esterification were determined by incubating hepatocytes with 0.4 mM albumin-bound [1-^{14}C]palmitate for 30 min [17]. For the determination of rates of fatty acid oxidation, reactions were started by the addition to cell incubations of [1-^{14}C]fatty acid (either palmitate or octanoate, 0.4 mM final concentration) bound to albumin, and carried out for 20 min [17].

2.3. Biochemical assays

ACC activity was determined in digitonin-permeabilized hepatocytes in an assay coupled to the fatty acid synthase reaction [16]. To measure enzyme activity, 100 μL of hepatocyte suspension was added to 100 μL of prewarmed digitonin-containing assay medium, and the reaction was carried out for 2 min, exactly as described in [16]. Fatty acid synthase (FAS) activity was monitored in digitonin-permeabilized hepatocytes as described previously [16]. Hepatocyte suspension (100 μL) was added to 100 μL of digitonin-containing assay medium, and the reaction was carried out for 4 min. CPT-I activity was determined in digitonin-permeabilized hepatocytes as the tetradecylglycidate (TDGA)-sensitive incorporation of radiolabeled L-carnitine into palmitoylcarnitine. In brief, hepatocytes were preincubated for 20 min in the absence or presence of 5 μM TDGA, a potent and specific inhibitor of CPT-I. Aliquots (100 μL) of both sets of hepatocyte incubations were added to 100 μL of prewarmed digitonin-containing assay medium, and the reaction was carried out for 40 s [18].

Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymatic method [18]. Rates of cholesterol synthesis *de novo* were determined as the incorporation of [1-^{14}C]acetate into non-saponifiable sterols extracted with light petroleum ether [16]. Nucleotide levels were determined in neutralized perchloric acid cell extracts by HPLC exactly as described by Gualix *et al.* [19]. Protein was determined by the method of Lowry *et al.* [20].

2.4. Statistical analysis

Results shown are the means ± SD of the number of animals indicated in every case. Cell incubations and/or enzyme assays were always carried out in triplicate. Statistical analysis was performed by Student's *t*-test.

3. Results and discussion

Incubation of hepatocytes with iodotubercidin markedly decreased the rate of fatty acid synthesis *de novo* and the activity of acetyl-CoA carboxylase, but the activity of fatty acid synthase was not changed by this treatment (Table 1). A direct effect of Itu on ACC can be ruled out since the activity of this enzyme was unaffected by addition of Itu up to 100 μM. Moreover, this effect of Itu on the activity of ACC was not mimicked by propentofylline (an inhibitor of adenosine transport into cells), neither blocked by A_2-adenosine receptor antagonist DMPX (results not shown). Taken together, these results indicate that under these conditions, ACC is a key regulatory activity of the fatty-acid-synthesizing process and may be a target for the effects of Itu on fatty acid synthesis. In addition, these effects are apparently not mediated by adenosine, and they should be unrelated to the effect of Itu on the release of adenosine by hepatocytes [21].

Iodotubercidin is a potent inhibitor of adenosine kinase that may alter the levels of adenine nucleotides in the cell. Thus, we determined these intracellular concentrations in

Table 1
Effects of iodotubercidin and AICAR on hepatic synthesis of fatty acids and cholesterol

Additions	Fatty acid synthesis (% of control)	ACC activity (% of control)	FAS activity (% of control)	Cholesterol synthesis (% of control)
None	100	100	100	100
20 μM Itu	32.5 ± 3.5**	46.2 ± 5.6*	93.2 ± 8.3	55.4 ± 6.8**
0.5 mM AICAR	5.3 ± 0.8*	0	95.6 ± 6.5	2.1 ± 0.5*
20 μM Itu + 0.5 mM AICAR	38.4 ± 6.7**	53.7 ± 9.0**	94.1 ± 7.9	50.1 ± 4.7**

Hepatocytes were incubated in the presence of indicated additions. Control (100%) values were: rates of synthesis of fatty acids and cholesterol, 30.5 ± 4.2 and 2.55 ± 0.26 nmol acetyl units/hr/mg cellular protein, respectively; ACC and FAS activities, 0.70 ± 0.06 and 1.0 ± 0.02 nmol product/min/mg cellular protein, respectively. Results are expressed as percent of control without additions and represent means ± SD of four different animals. Significantly different from incubations with no additions: $^{*}P < 0.001$; $^{**}P < 0.01$.

hepatocytes incubated in the absence and in the presence of 20 μM Itu. ATP and AMP concentrations were, respectively, 2.25 ± 0.33 and 0.27 ± 0.05 μmol/g cell wet mass in control incubations, and 1.51 ± 0.10 and 0.39 ± 0.06 μmol/g cell wet mass, respectively, for ATP and AMP in hepatocytes incubated with Itu. Thus, addition of Itu caused an important decrease in ATP concentration, and a concomitant smaller increase in AMP concentration, so that the AMP/ATP ratio increased more than two times. These nucleotide levels and changes in ATP concentration caused by Itu were similar to those reported by others [3,4,21,22]. These changes may be the result of two interacting reactions. Firstly, the presence of a very active adenylyl kinase makes AMP and ATP concentrations tend to change in opposite directions to maintain the reaction close to equilibrium [11,12]. On the other hand, studies on hepatic metabolism of adenine nucleotides suggest the existence of a substrate cycle between AMP and adenosine [21], that can be interrupted by inhibition of adenosine kinase by Itu.

The increase in the AMP/ATP ratio could result in stimulation of AMP-activated protein kinase and modulation of AMPK-regulated activities. AICAR is a selective cell-permeable activator of AMPK [10,23]. Thus, we compared the effects of Itu and AICAR on the syntheses of fatty acids and cholesterol in rat hepatocytes (Table 1). Incubation with Itu decreased the activity of ACC and the rates of synthesis of fatty acids and cholesterol. However, these effects were much more potent for AICAR that almost completely inhibited these AMP-regulated activities. Also evident is the fact that simultaneous addition of Itu and AICAR almost exactly matched the effect of Itu alone, probably because Itu inhibits adenosine kinase and blocks the conversion of AICAR into 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), the structural analogue of AMP which mimics its effect on the activation of AMPK [22,23]. Taken together, these data afford indirect evidence to support the notion that incubation with Itu increases AMP/ATP ratio and may lead to partial activation of AMPK [12], but not so strong as that produced by accumulation of ZMP in hepatocytes incubated with 0.5 mM AICAR.

Modulation of acetyl-CoA carboxylase is also essential for the control of CPT-I activity and fatty acid oxidation in hepatocytes. The rate of palmitate oxidation was enhanced by the addition of Itu to hepatocyte incubations, but octanoate oxidation was not affected by Itu (Table 2). It is well established that palmitate is transported into mitochondria by a carnitine-dependent process, whereas octanoate may enter mitochondria independently of carnitine [14,15]. Therefore, this result suggests that the rate of the reaction catalyzed by CPT-I might be increased upon addition of Itu to the incubation of hepatocytes. However, when CPT-I activity was determined in hepatocytes permeabilized with digitonin, this activity was not significantly altered by incubation with Itu (Table 2). At this regard it should be pointed out that in order to measure enzyme activity, the plasma membrane is permeabilized and this causes the cytosol to leak out of the cell, leading to a large dilution of cytosolic components, including malonyl-CoA [18,24]. Inhibition of CPT-I activity by malonyl-CoA is a well-described property of the enzyme [14,15]. Hence, we determined the effect of Itu on malonyl-CoA levels in hepatocytes. In line with the iodotubercidin-mediated inhibition of ACC (Table 1), Itu induced a marked decrease in the intracellular concentration of malonyl-CoA (Table 2). In addition, based on determinations of CPT-I activity at different times after cell permeabilization

Table 2
Effects of iodotubercidin on the rates of fatty acid oxidation and esterification, CPT-I activity and malonyl-CoA concentration

Parameter	Additions	
	None	20 μM Itu
Octanoate oxidation (N = 4)	67.9 ± 1.6	64.9 ± 3.40
Palmitate oxidation (N = 5)	41.5 ± 5.2	58.6 ± 4.2*
CPT-I activity (N = 5)	0.89 ± 0.06	1.00 ± 0.09
Malonyl-CoA concentration (N = 4)	0.091 ± 0.009	0.035 ± 0.001*
Palmitate esterification (N = 4)		
Triacylglycerols	40.2 ± 4.9	23.6 ± 0.8*
Phospholipids	17.4 ± 1.0	15.4 ± 0.5**

Rates of fatty acid oxidation and esterification are expressed as nmol fatty acid converted into product/hr/mg cellular protein. Activities of CPT-I are expressed as nmol product/min/mg cellular protein. Malonyl-CoA concentrations are in nmol/mg cellular protein. Data are means ± SD of the number of animals given in parentheses. $^{*}P < 0.01$ and $^{**}P < 0.05$, relative to incubations with no additions.

(not shown), we came to the conclusion that in our system, a malonyl-CoA-independent mechanism is not involved in activation of CPT-I [25,26]. Therefore, the increased rate of palmitate oxidation in hepatocytes incubated with Itu may well be attributed to this decrease of malonyl-CoA concentration and the concomitant relief of the inhibition of CPT-I.

Taken together, our results allow us to conclude that Itu markedly affects hepatic fatty acid metabolism by stimulating mitochondrial fatty acid oxidation at the expense of lipogenesis. We have identified acetyl-CoA carboxylase as the target enzyme for these effects of Itu. Moreover, these data afford indirect evidence which can be taken to suggest a mechanism responsible for this cellular response, based on the increase of AMP/ATP ratio and the concomitant stimulation of the AMPK. Clearly, further research is required to elucidate fully this suggested mechanism of action of Itu, and studies investigating this issue are in progress. In addition, these studies may contribute to a better understanding of this widely used inhibitor of adenosine kinase. Interestingly, Itu has been shown to inhibit partially purified AMPK, but only in the absence of AMP [7], therefore, not likely in isolated hepatocytes.

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