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Effect of hypoxia on phosphatidylcholine biosynthesis in the isolated hamster heart

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In hamster heart, the majority of the phosphatidylcholine is synthesized via the CDP-choline pathway, and the rate-limiting step of this pathway is catalysed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15). We have shown previously [Choy (1982) J. Biol. Chem. **257**, 10928–10933] that, in the myopathic heart, the level of cardiac CTP was diminished during the development of the disease. In order to maintain the level of CDP-choline, and consequently the rate of phosphatidylcholine biosynthesis, cardiac cytidylyltransferase activity was increased. However, it was not clear if the same compensatory mechanism would occur when the cardiac CTP level was decreased rapidly. In this study, hypoxia of the hamster heart was produced by perfusion with buffer saturated with 95% N_2. The heart was pulse-labelled with radioactive choline and then chased with non-radioactive choline for various periods under hypoxic conditions. There was a severe decrease in ATP and CTP levels within 60 min of hypoxic perfusion, with a corresponding fall in the rate of phosphatidylcholine biosynthesis. Analysis of the choline-containing metabolites revealed that the lowered ATP level did not affect the phosphorylation of choline to phosphocholine, but the lower CTP level resulted in the decreased conversion of phosphocholine to CDP-choline. Determination of enzyme activities revealed that hypoxic treatment resulted in the enhanced translocation of cytidylyltransferase from the cytosolic to the microsomal form. This enhanced translocation was probably caused by the accumulation of fatty acids in the heart during hypoxia. We postulate that the enhancement of translocation of the cytidylyltransferase to the microsomal form (a more active form) is a mechanism by which the heart can compensate for the decrease in CTP level during hypoxia in order to maintain phosphatidylcholine biosynthesis.

INTRODUCTION

Phosphatidylcholine is an important structural and functional membrane component in mammalian organs [1,2]. It is the principal phospholipid in the mammalian heart, comprising approx. 40% of the total membrane phospholipids [3]. In the hamster heart the majority of phosphatidylcholine is synthesized via the CDP-choline pathway [4,5]. Choline is actively taken up by the heart [5–7] and rapidly phosphorylated by ATP to phosphocholine. Phosphocholine is then converted to CDP-choline by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) which requires CTP as cofactor [4,5]. This reaction is generally regarded as the rate-limiting step in the CDP-choline pathway [5,8]. Finally, CDP-choline is condensed with 1,2-diacylglycerol for the formation of phosphatidylcholine.

The requirement for ATP and CTP in the CDP-choline pathway suggests that the intracellular levels of these compounds may affect the rate of phosphatidylcholine biosynthesis [8]. We have shown previously that an increase in CTP levels in polio-infected HeLa cells caused an enhancement of phosphatidylcholine biosynthesis [9]. Alternatively, a 34% decrease in levels of both ATP and CTP in the hearts of 150–200-day-old myopathic hamsters [10] caused a decrease in the labelling of phosphatidylcholine. The decrease in ATP level had no immediate effect on the phosphorylation of choline, but the diminished level of CTP caused a decrease in the conversion of labelled phosphocholine to CDP-choline. In order to prevent a net decrease in phosphatidylcholine biosynthesis, CTP:phosphocholine cytidylyltransferase activity in the myopathic heart was elevated, which acted as a compensatory mechanism for maintaining a functional level of CDP-choline. At present, the mechanism for triggering the increase in cytidylyltransferase activity is undefined. Since the decrease in CTP levels in the myopathic heart was instilled over a prolonged period, it was not clear whether the increase in cytidylyltransferase activity could be triggered by a rapid decrease in ATP and CTP levels. The isolated heart perfused under hypoxic conditions is an established model for decreasing the overall oxygen delivery to below the critical level required to support the metabolic ATP demands of the tissue [11,12] and is the model of choice for this study. Since ATP is a precursor for biosynthesis of CTP, perfusion under hypoxic conditions may also decrease the cardiac CTP level. In this study, we confirm the fall in levels of ATP and CTP in the heart under hypoxia, and we examine the effects of hypoxia on cardiac phosphatidylcholine biosynthesis.

MATERIALS AND METHODS

Materials

[*Me*-^{3}H]Choline, [*Me*-^{14}C]choline. CDP-[*Me*-^{14}C]choline and [γ-^{32}P]ATP were obtained from New England Nuclear. Phospho[*Me*-^{3}H]choline was synthesized enzymically from [*Me*-^{3}H]choline by the procedure of Paddon & Vance [13]. Aqueous counting scintillant was obtained from Amersham Corporation. T.l.c. plates (Sil-G25) were obtained from Brinkman Inc. BF_3/methanol kit and Celite 545 were obtained from Supelco Laboratory. A free-fatty-acid test kit was obtained from Boehringer-Mannheim. Ion-exchange resin (AG1-X8) was obtained from Bio-Rad. Activated charcoal was obtained from Sigma. All other biochemicals were of analytical grade and were purchased from either Sigma or Fisher.

Syrian golden hamsters weighing 100 ± 15 g were used throughout the study. The hamsters were sustained on Purina hamster chow and tap water, *ad libitum*, in a light- and temperature-controlled room.

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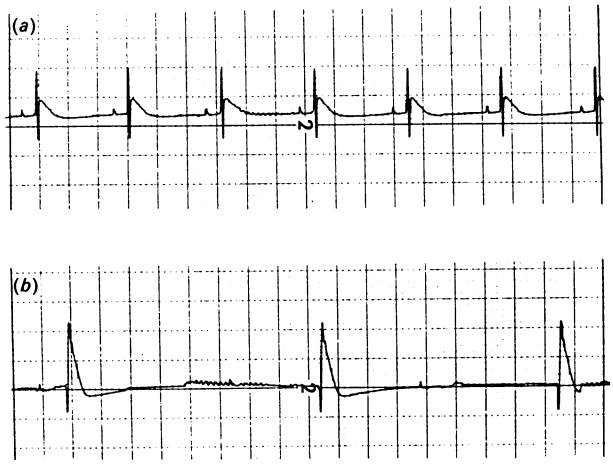

Fig. 1. Electrocardiac recordings of isolated hamster heart perfused under hypoxic conditions

Electrocardiac recordings were obtained at 60 min of perfusion by placing one electrode on the aorta and another electrode in the buffer bathing the apex of the heart. (*a*) Hearts perfused with 95%-O_2-saturated buffer (control); (*b*) hearts perfused with 95%-N_2-saturated buffer (hypoxic).

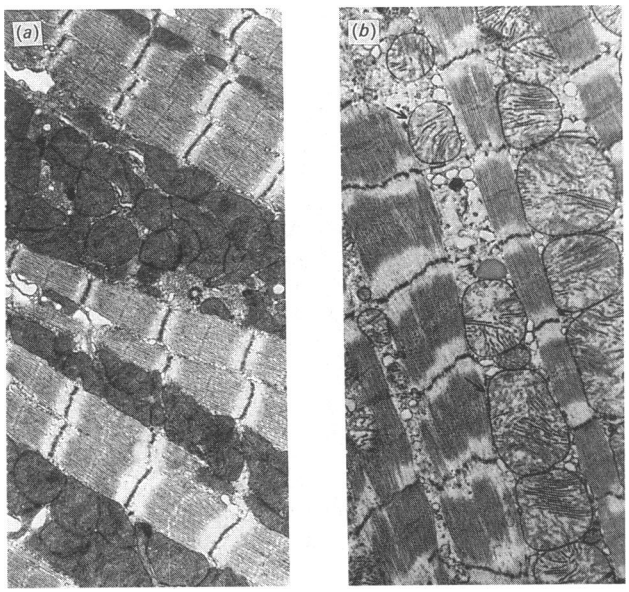

Fig. 2. Electron micrographs of hamster heart perfused under hypoxic conditions

Samples from hamster hearts were analysed after 60 min of perfusion. (*a*) Hearts perfused with 95%-O_2-saturated buffer (control); (*b*) hearts perfused with 95%-N_2-saturated buffer (hypoxic). Magnification ×13510.

Perfusion of hamster hearts

Hamster hearts were perfused in the Langendorff mode [14] with Krebs–Henseleit buffer [15] as described previously [5]. Under these conditions the hearts remain viable for at least 4 h of perfusion [16]. The hearts were pulse-labelled for 30 min with 95%-O_2-saturated buffer containing 0.01 mM-[*Me*-^{3}H]choline. Subsequently, hearts were perfused (chased) with the 95%-O_2-saturated buffer (control) or 95%-N_2-saturated buffer (hypoxic) containing 0.01 mM-choline for 30, 45 or 60 min. The P_{O_2} of the 95%-O_2-saturated buffer was 522±15 mmHg (69.6±2.0 kPa) and the P_{O_2} of the 95%-N_2-saturated buffer was 22±5 mmHg (2.9±0.7 kPa). The viability of the heart during perfusion was monitored by electrocardiac recording. An electrode was placed on the aorta and another electrode was immersed in the buffer solution bathing the apex of the heart. The electrocardiogram was found to differ between hearts perfused with 95%-O_2-saturated buffer (Fig. 1*a*) and those perfused with 95%-N_2-saturated buffer (Fig. 1*b*). Perfusion of the heart with 95%-N_2-saturated buffer for 60 min caused a complete atrioventricular block [17]. However, subsequent perfusion with the 95%-O_2-saturated buffer for another 60 min resulted in an electrocardiogram which resembled the control, indicating that the electrophysiological abnormalities generated under hypoxic insult were reversible. Further hypoxic exposure (> 60 min) caused non-reversible electrophysiological damage. Thus all experiments in this study were limited to hypoxic exposure for 60 min or less. Histological analysis of the ventricular tissue by electron microscopy revealed some degree of mitochondrial swelling (arrows) after 60 min of perfusion with 95%-N_2-saturated buffer (Fig. 2*b*) when compared with the control (Fig. 2*a*). The mitochondrial swelling was shown to be reversible upon re-oxygenation [18]. There were no significant changes in the morphology of the other organelles.

Determination of radioactivity incorporation into choline-containing metabolites

Subsequent to perfusion, the hearts were homogenized in 20 ml of chloroform/methanol (1:1, v/v). A portion of the homogenate was taken for determination of total radioactivity. The homogenate was then allowed to separate into two phases by the addition of chloroform and 0.05 M-KCl until a 4:2:3 (by vol.) ratio of chloroform/methanol/0.05 M-KCl was obtained. The phosphatidylcholine fraction in the organic phase was separated from the other phospholipids by t.l.c. with a solvent system containing chloroform/methanol/water/NH_4OH (70:30:4:1, by vol.). The choline-containing metabolites in the aqueous phase were separated by t.l.c. using a solvent system containing methanol/0.6% NaCl/NH_4OH (10:10:1, by vol.).

Determination of enzyme activities

Subsequent to perfusion, the hearts were weighed and a 20% (w/v) homogenate in 0.145 M-NaCl/5 mM-Tris/HCl (pH 7.4) was prepared. The post-mitochondrial fraction was obtained by centrifugation at 12000 *g* for 15 min, and was centrifuged at 105000 *g* for 60 min to yield the cytosolic and the crude microsomal fractions. The crude microsomal fraction was washed once and recentrifuged at 105000 *g* for 60 min, and the pellet was resuspended in 1 ml of buffer. The protein contents of homogenate, microsomal suspension and cytosol were determined by the method of Lowry *et al.* [19].

Glucose-6-phosphatase, a microsomal marker, was assayed as described by Moore [20]. Choline kinase activity was assayed in the cytosolic fraction using [*Me*-^{3}H]choline as substrate [21]. CTP:phosphocholine cytidylyltransferase activity was assayed in the cytosolic and microsomal fractions using phospho[*Me*-^{3}H]choline [22]. In some of the cytidylyltransferase assays, total hamster liver lipid extract (1 mg of phospholipid/ml of reaction mixture) was added to the cytosolic fraction prior to assay to obtain maximal stimulation of the enzyme [23]. CDP-choline:1,2-diacylglycerol cholinephosphotransferase was assayed in the microsomal fraction using CDP-[*Me*-^{14}C]choline [16]. Phosphocholine phosphatase activity was assayed in the microsomal fraction using phospho[*Me*-^{3}H]choline [24]. The metabolic fate of the labelled CDP-choline was determined as described previously [10].

Determination of choline, phosphocholine and CDP-choline pool sizes

Three groups of hamster hearts were employed for this study: (1) non-perfused hearts, (2) hearts perfused with 95%-O_2-saturated buffer and (3) hearts perfused with 95%-N_2-saturated buffer. Immediately after perfusion the hearts were frozen in liquid N_2. Subsequently the hearts were weighed and homogenized in 20 ml of chloroform/methanol (1:1, v/v). Water (10 ml) was added to the homogenate to cause phase separation. The aqueous phase was removed and the solvent was evaporated under reduced pressure. The recovery of the choline-containing metabolites was estimated by the addition of [*Me*-^{3}H]choline, phospho[*Me*-^{3}H]choline and CDP-[*Me*-^{14}C]choline to the extracts.

The quantification of choline-containing metabolites was performed as described previously [25]. Briefly, the residue in the aqueous phase was redissolved in 10 ml of water and applied to a Dowex AG1-X8 (OH^- form) column (1 cm × 30 cm). Choline was eluted from the column with 60 ml of methanol/water (1:1, v/v) followed by 10 ml of water. Phosphocholine and CDP-choline were co-eluted with 200 ml of 0.4 M-NH_4HCO_3. The choline, phosphocholine and CDP-choline fractions were evaporated under reduced pressure. The fractions containing phosphocholine and CDP-choline were redissolved in 5 ml of water and applied to a Norite A/celite (1:1, v/v) column (1 cm × 5 cm). Phosphocholine was eluted from the column with 20 ml of water followed by 15 ml of 2% ethanol. CDP-choline was then eluted with 30 ml of 40% ethanol containing 1% NH_4OH. The phosphocholine fraction was digested with 10 units of *Escherichia coli* alkaline phosphatase (type II) to yield choline. The CDP-choline fraction was digested with 10 units of alkaline phosphatase and 10 units of *Crotalus adementeus* phosphodiesterase (type III) to yield choline.

The choline, digested phosphocholine and CDP-choline fractions were dissolved separately in 4 ml portions of water. The choline from each sample was extracted with 3 ml of heptan-3-one containing tetraphenylboron (10 mg/ml). The choline in heptan-3-one was back-extracted with 0.5 ml of 0.4 M-HCl followed by a second extraction with 3 ml of 1.0 M-HCl. The HCl extracts from each sample were combined and lyophilized. The yield was calculated from the percentage of radioactivity recovered in each sample. The amount of choline in each sample was determined by the quantitative conversion of choline to [γ-^{32}P]phosphocholine [26].

Determination of nucleotide levels in hamster hearts

After perfusion, the hearts were immediately frozen in liquid N_2 and stored at −70 °C until use. The hearts were then weighed and homogenized in 6 ml of chloroform/methanol (2:1, v/v). For recovery determination, labelled CTP was added to the homogenates. Water (3 ml) was added to the homogenate to cause phase separation. The aqueous phase was removed and the organic phase was washed twice with 3 ml of water. The aqueous phase extracts were pooled and concentrated by evaporation under reduced pressure. The nucleotide content in each sample was analysed by h.p.l.c. Specifically, a 20 μl sample was applied to an Ultrasphere-ODS reversed-phase column. Nucleotides were eluted from the column with 0.03 M-KH_2PO_4 containing 0.02 M-tetrabutylammonium phosphate in 19% acetonitrile, pH 2.65.

Determination of phospholipids and fatty acids

After perfusion, the hearts were homogenized in 20 ml of chloroform/methanol (2:1, v/v), and 0.05 M-KCl was added to the homogenate until a final ratio of chloroform/methanol/0.05 M-KCl of 4:2:3 (by vol.) was obtained. After phase separation, the organic phase was extracted twice with 10 ml of chloroform/0.05 M-KCl (1:1, v/v) and the extracts were pooled. The volume of the pooled extracts was decreased under N_2 and the phospholipid fractions in each extract were separated by t.l.c. The phosphorous content of each phospholipid fraction was determined by the method of Bartlett [27].

After perfusion, hearts from each group were pooled and homogenized, and subcellular fractions were prepared. Fatty acids were extracted from the cytosol [28] and determined quantitatively by the method of Shimizu *et al.* [29]. The acyl composition of the cytosolic fatty acids was analysed by g.l.c. as described by Metcalfe & Schnitz [30].

Translation of cytidylyltransferase *in vitro*

Hamster hearts were perfused for 60 min with 95%-O_2-saturated buffer or 95%-N_2-saturated buffer. After perfusion, heats were homogenized in 0.145 M-NaCl/5 mM-Tris/HCl (pH 7.4), and the post-mitochondrial fraction was obtained by centrifugation at 12000 ***g*** for 15 min. This fraction was then centrifuged at 105000 ***g*** for 60 min and the resulting supernatant was designated the cytosolic fraction. A 2 ml sample of cytosolic fraction from control hearts or from hearts perfused under hypoxic conditions was incubated at 37 °C for 15 min with 1.5 ml of post-mitochondrial supernatant prepared from the homogenate of non-perfused hearts. The mixture was centrifuged at 105000 ***g*** for 60 min. The pellet was resuspended in 1.5 ml of homogenizing buffer and designated the microsomal suspension, and the supernatant from this centrifugation was designated the cytosolic suspension.

Statistical analysis of data

Results in this study are depicted as means ± S.D. (number of experiments), unless otherwise noted. A two-tailed Student's *t* test was used for the determination of significance. The level of significance was defined as $P < 0.05$.

RESULTS

Effect of hypoxia on phosphatidylcholine biosynthesis

Hamster hearts were perfused with [*Me*-^{3}H]choline for 30 min in 95%-O_2-saturated Krebs–Henseleit buffer. Subsequently, the hearts were perfused with 95%-N_2-saturated buffer for 30, 45 and 60 min. In another set of experiments, hamster hearts perfused with 95%-O_2-saturated buffer after pulse-labelling were used as controls. There was no significant difference in the amount of labelling of phosphatidylcholine between the hearts perfused with 95%-N_2-saturated buffer (hypoxic) and with 95%-O_2-saturated buffer (control) during 30 or 45 min of chase (Fig. 3). Perfusion of hamster hearts for 60 min under hypoxic conditions did not change the total amount of radioactivity in the heart, but resulted in a decrease in the labelling of phosphatidylcholine (Fig. 3). Analysis of the choline-containing metabolites revealed that there was an accumulation of labelled phosphocholine during hypoxic treatment, with a simultaneous decrease in the labelling of CDP-choline; no change in the labelling of choline was observed at any time of perfusion (Fig. 4). The accumulation of radioactivity in the phosphocholine fraction quantitatively accounted for the decrease in radioactivity in the CDP-choline and phosphatidylcholine fractions (Figs. 3 and 4). In the hypoxic and control hearts, the sum of radioactivities recovered from the choline, phosphocholine, CDP-choline and phosphatidylcholine fractions accounted for over 98% of the total radioactivity in the homogenate. Significant but equal amounts of labelled choline were found in the perfusate of both groups of hearts during the chase. This is not surprising,

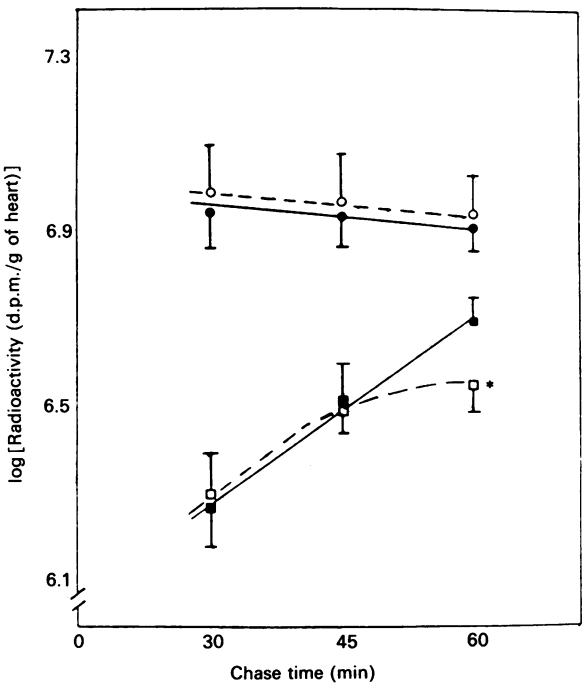

Fig. 3. Radioactive choline uptake and its incorporation into phosphatidylcholine in hamster hearts perfused under hypoxic conditions

Hamster hearts were perfused for 30, 45 and 60 min with 95%-O_2-saturated buffer (control) or 95%-N_2-saturated buffer (hypoxic). The total uptake of radioactivity by the heart (○, ●) and radioactivity incorporated into phosphatidylcholine (□, ■) at each time point were determined. The open symbols indicate hearts perfused under hypoxic conditions and the closed symbols represent controls. Each time point is the mean of three separate experiments. The vertical bars are S.D. values; * $P < 0.05$ compared with control.

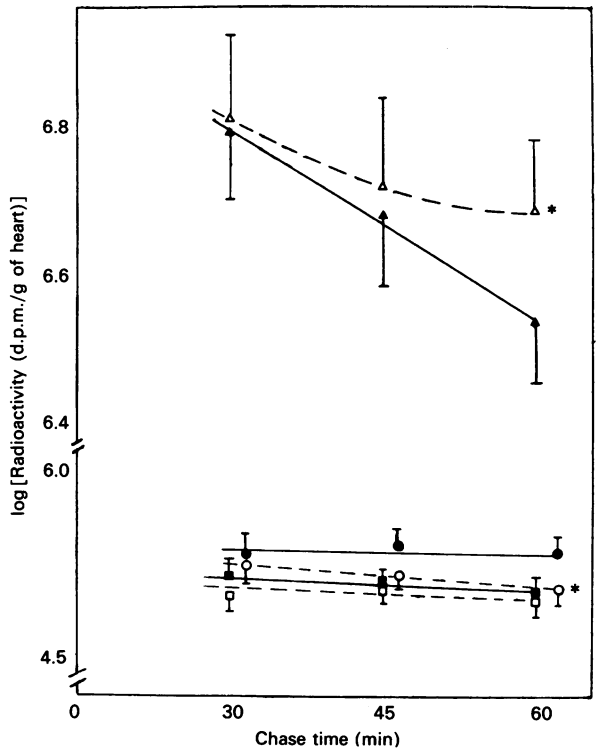

Fig. 4. Radioactivity incorporated into choline-containing metabolites in hamster hearts perfused under hypoxic conditions

Hamster hearts were perfused for 30, 45 and 60 min with 95%-O_2-saturated buffer (control) or 95%-N_2-saturated buffer (hypoxic). Radioactivity incorporated into the choline (□, ■), phosphocholine (△, ▲) and CDP-choline (○, ●) fractions was determined. The open symbols indicate hearts perfused under hypoxic conditions and the closed symbols represent controls. Each time point is the mean of at least three separate experiments and the vertical bars are the S.D. values; * $P < 0.05$ compared with control.

since the loss of labelled choline into the perfusate has been demonstrated previously [5].

Effect of hypoxia on the enzymes of the CDP-choline pathway

The specific activities (nmol of product formed/min per mg of protein) of the enzymes in the CDP-choline pathway in the control and hypoxic hearts were examined. At 30 min of perfusion, no significant differences in the specific activities of choline kinase, cytidylyltransferase and cholinephosphotransferase were detected between the control and hypoxic hearts (results not shown). At 60 min of perfusion, there was no difference in the specific activities of choline kinase and cholinephosphotransferase between the two groups. In addition, the specific activity of phosphocholine phosphatase (for the hydrolysis of phosphocholine) was not altered (Table 1). The specific activity of microsomal cytidylyltransferase was found to have increased at 60 min of hypoxic perfusion, but no significant change was detected in the cytosolic fraction. This is not surprising, since the cytosolic cytidylyltransferase requires lipids for maximum activity [28,31]. Upon activation, the specific activity of the cytosolic enzyme in the hypoxic heart was found to be decreased when compared with the control. In order to determine whether there was a redistribution of the cytidylyltransferase from the cytosolic to the microsomal fraction at 60 min of hypoxia, the total cytidylyltransferase activity (nmol of product formed/min per g of heart) in both the cytosolic and microsomal fractions was estimated. The total microsomal enzyme activity was calculated based on the yield of microsomes from the homogenate. The total cytosolic enzyme activity was calculated based on the complete activation of the enzyme in the presence of lipids. Under hypoxic conditions, the activity of cytidylyltransferase was found to increase in the microsomal fraction with a corresponding decrease in the cytosolic fraction (Table 1). The sum of the enzyme activities in these two fractions was similar to that obtained in the control. These results clearly indicate that a redistribution of cytidylyltransferase occurred in hearts perfused under hypoxic conditions. Since the microsomal form of cytidylyltransferase has been regarded as the active form of the enzyme [32–34], an increase in the microsomal enzyme activity would imply a corresponding increase in CDP-choline synthesis. In view of the fact that the labelling of CDP-choline was actually decreased during hypoxic treatment, such a decrease cannot be explained by the translocation and/or activation of the cytidylyltransferase.

Pool sizes of choline-containing metabolites in the CDP-choline pathway

Changes in the pool sizes of the choline-containing metabolites in the CDP-choline pathway might affect the specific radioactivities of these intermediates, which may subsequently affect the labelling of phosphatidylcholine. Thus the pool sizes of choline, phosphocholine, CDP-choline and phosphatidylcholine were determined in the unperfused heart and in hearts perfused with 95%-O_2-saturated buffer or 95%-N_2-saturated buffer. As seen in Table 2, there were no significant differences in the pool sizes of the choline-containing metabolites between the three groups of hearts. Clearly, the decrease in labelling of phospha-

tidylcholine in hearts perfused under hypoxic conditions was not caused by pool size changes of the choline-containing metabolites.

Effect of hypoxia on CDP-choline metabolism and nucleotide content

Since CDP-choline is the immediate precursor of phosphatidylcholine, the cause of the decrease in labelling of CDP-choline in hearts perfused under hypoxic conditions was further investigated. One possible explanation for the decrease in the labelling of CDP-choline was that the catabolism of this compound might be different under hypoxic conditions. In order to examine this possibility, homogenates from control hearts and from hearts perfused under hypoxic conditions were incubated with labelled CDP-choline. The results obtained indicate that CDP-choline was not catabolized differently in the hypoxic and the control hearts.

Since ATP and CTP are the known cofactors for phosphatidylcholine biosynthesis via the CDP-choline pathway, the levels of these nucleotides in hearts perfused under different conditions were compared. The levels of both ATP and CTP were found to progressively decrease under hypoxic conditions (Table 3). It is interesting to note that, in spite of the diminished level of ATP (39% of control) in the hypoxic heart, the conversion of choline to phosphocholine was not affected. However, a severe fall in CTP (28% of control) appears to be one of the factors which decreased the conversion of labelled phosphocholine to CDP-choline and, consequently, resulted in the accumulation of radioactivity in phosphocholine.

Effect of hypoxia on phospholipid and fatty acid content

The mechanism for the translocation/activation of cytidylyltransferase in the microsomal fraction of the heart perfused under hypoxic conditions was investigated. Cytidylyltransferase has been shown to be activated by a number of lipids [23,31,35,36], and some of these lipids also cause the translocation of the enzyme from the cytosolic to the microsomal compartment. In this study, the hearts were perfused with 95%-O_2-saturated buffer or 95%-N_2-saturated buffer for 60 min, and the cardiac phospholipid contents after perfusion were determined. There was no significant difference in phospholipid content between the two experimental groups (Table 4). Hence the increase in microsomal cytidylyltransferase activity under hypoxic conditions could not be attributed to a change in the cardiac phospholipid content.

Fatty acids have been shown to activate cytidylyltransferase

Table 1. Activities of the enzymes of the CDP-choline pathway and of phosphocholine phosphatase in control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95%-O_2-saturated buffer (control) or 95%-N_2-saturated buffer (hypoxic). After perfusion, enzyme activities in the appropriate subcellular fraction were determined as described in the Materials and methods section. * $P < 0.05$ compared with control.

Enzyme	Activity (nmol/min per mg of protein)	
	Control	Hypoxic
Choline kinase (cytosol)	0.52±0.09 (4)	0.47±0.09 (4)
Phosphocholine transferase (microsomal)	1.04±0.04 (4)	1.01±0.09 (4)
Phosphocholine cytidylyltransferase (microsomal)	0.63±0.24 (11)	1.31±0.52 (10)*
Phosphocholine cytidylyltransferase (cytosol)	0.57±0.21 (11)	0.48±0.24 (10)
Phosphocholine cytidylyltransferase (cytosol + lipid)	1.76±0.29 (11)	1.23±0.24 (10)*
Phosphocholine phosphatase (microsomal)	2.94±0.17 (3)	2.88±0.10 (3)
	Total activity (nmol/min per g of heart)	
Phosphocholine cytidylyltransferase (microsomal)	9.30±2.86 (11)	16.63±6.59 (10)*
Phosphocholine cytidylyltransferase (cytosol)	23.04±3.37 (11)	16.31±3.66 (10)*

Table 3. ATP and CTP concentrations in control and hypoxic hearts

Hamster hearts were perfused for 30 or 60 min with 95%-O_2-saturated buffer (control) or 95%-N_2-saturated buffer (hypoxic). After perfusion ATP and CTP levels were determined as described in the Materials and methods section. * $P < 0.05$ compared with control.

Time	[ATP] (μmol/g of heart)		[CTP] (nmol/g of heart)	
	Control	Hypoxic	Control	Hypoxic
30 min	2.70±0.79 (3)	1.27±0.41 (3)*	11.80±1.84 (3)	7.03±0.55 (3)*
60 min	2.54±0.16 (3)	0.99±0.09 (3)*	11.49±2.24 (3)	3.25±1.12 (3)*

Table 2. Choline, phosphocholine, CDP-choline and phosphatidylcholine contents in control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95%-O_2-saturated buffer or 95%-N_2-saturated buffer. The pool sizes of the choline-containing metabolites and phosphatidylcholine were determined as described in the Materials and methods section.

	Pool size (nmol/g of heart)		
	Before perfusion	Perfusion with 95%-O_2-saturated buffer	Perfusion with 95%-N_2-saturated buffer
Choline	151±10 (3)	137±21 (4)	141±11 (3)
Phosphocholine	230±20 (3)	203±19 (3)	201±24 (4)
CDP-choline	100±12 (4)	106±8 (4)	105±25 (4)
Phosphatidylcholine*	12.73±0.84 (3)*	13.14±1.39 (3)*	12.85±1.12 (3)*

* Phosphatidylcholine pool size is given as μmol of lipid phosphate/g of heart.

Table 4. Phospholipid content in control and hypoxic hearts

Hamster hearts were perfused for 60 min with 95%-O_2-saturated buffer (control) or 95%-N_2-saturated buffer (hypoxic). After perfusion, the phospholipid content was determined as described in the Materials and methods section.

Phospholipid species	Content (μmol of lipid P_i/g of heart)	
	Control	Hypoxic
Phosphatidylcholine	13.14±1.39(3)	12.85±1.12(3)
Phosphatidylethanolamine	10.41±1.18(3)	9.61±0.55(3)
Sphingomyelin	1.85±0.09(4)	1.97±0.23(4)
Lysophosphatidylcholine	0.18±0.07(4)	0.16±0.06(4)
Lysophosphatidylethanolamine	0.12±0.04(4)	0.13±0.03(4)
Phosphatidylserine + phosphatidylinositol	1.32±0.06(4)	1.41±0.17(4)
Phosphatidylglycerol + cardiolipin	3.58±0.69(4)	3.74±0.62(4)

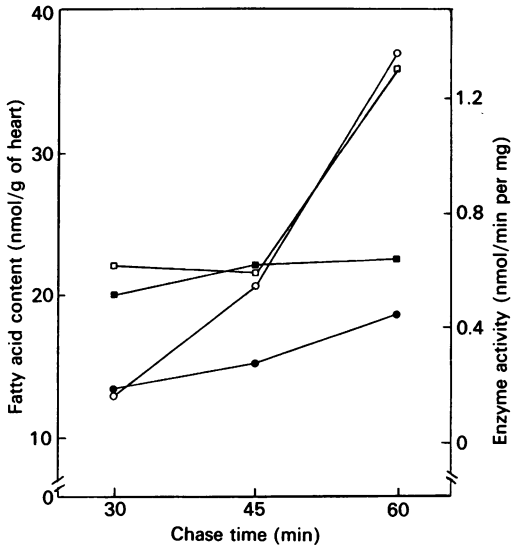

Fig. 5. CTP:phosphocholine cytidylyltransferase activity and cytosolic fatty acid content in hearts perfused under hypoxic conditions

Hamster hearts were perfused for 30, 45 and 60 min with 95%-O_2-saturated buffer (control) or 95%-N_2-saturated buffer (hypoxic). Cytidylyltransferase activity in the microsomal fraction (□, ■) and cytosolic fatty acid content (○, ●) were determined at each time point. The open symbols indicate hearts perfused under hypoxic conditions and the closed symbols are controls. Each point represents the mean of at least two separate experiments.

[36] and some fatty acids ($> C_{8:0}$) may also promote the translocation of the enzyme from the cytosolic to the microsomal fraction [37]. Hence the fatty acid contents in the homogenate and the cytosol of controls and of hearts perfused under hypoxic conditions were determined. No significant change in total fatty acid content of the homogenates was detected in the heart up to 60 min of hypoxic perfusion. However, the cytosolic fatty acid content of the hypoxic heart was elevated by 1.4- and 2.0-fold at 45 and 60 min of hypoxia respectively (Fig. 5). The 2-fold increase in the cytosolic fatty acid content correlated well with the increase in microsomal cytidylyltransferase activity. In order to determine the type of fatty acid responsible for the increase in enzyme activity, the cytosolic fatty acid composition at 60 min of hypoxia was analysed and compared with that in the control. No change in the percentage distribution of the fatty acid species was

Table 5. Translocation of cytidylyltransferase *in vitro*

Hamster hearts were perfused for 60 min with 95%-O_2-saturated buffer or 95%-N_2-saturated buffer. Cytosolic fractions were prepared from the tissue homogenates after perfusion, and aliquots (2 ml) of the cytosol were incubated with 1.5 ml of post-mitochondrial fraction prepared from normal hamster heart at 37 °C for 15 min. After incubation, the mixture was centrifuged at 100000 *g* for 60 min. Cytidylyltransferase activities were determined in the supernatant (cytosolic fraction) in the presence of lipids, and in the pellet suspended in 1.5 ml of buffer (microsomal suspension) as described in the Materials and methods section. Values represent the means of two separate experiments.

	Cytidylyltransferase activity (nmol/min per ml)	
	Control	Hypoxic
Microsomal suspension	0.74	0.90
Cytosolic fraction	0.91	0.74

detected between the two groups. The higher fatty acid content in the cytosolic fraction of hearts perfused under hypoxic conditions was attributed to a general increase in all acyl species.

Translocation of cytidylyltransferase *in vitro*

The data from the preceding sections indicate that the translocation of cytidylyltransferase during hypoxia might be caused by an increase in cytosolic fatty acid content. In order to verify this hypothesis, cytosol from hearts perfused under hypoxic conditions was incubated with a post-mitochondrial fraction prepared from a non-perfused heart. Cytosol prepared from hearts perfused with oxygenated buffer was used as a control. After incubation, the mixture was centrifuged at 105000 *g* for 60 min. Cytidylyltransferase activities were determined in the supernatant (cytosolic fraction) and the pellet suspension (microsomal fraction), and the results are depicted in Table 5. Upon incubation with the cytosolic fraction from the hypoxic heart, a 22% increase in total microsomal cytidylyltransferase activity was observed, with a corresponding 19% decrease in total cytosolic enzyme activity (when assayed with lipid activators).

DISCUSSION

The objective of the present study was to investigate the effect of rapid energy depletion on phosphatidylcholine biosynthesis in the heart, and to identify the adaptive changes that occurred for maintaining phosphatidylcholine biosynthesis. In order to produce a rapid decrease in the high energy triphosphate nucleotides in the heart, the hypoxic model was employed [11,12]. Perfusion under hypoxic conditions for up to 60 min causes some electrophysiological and morphological changes which are reversible upon reoxygenation. Perfusion under hypoxic conditions for more than 60 min would inevitably produce further decreases in ATP and CTP levels, but would also generate irreversible damage to the cardiac tissue [38].

A significant decrease in cardiac ATP was observed at 30 and 60 min of hypoxic perfusion, but the conversion of choline to phosphocholine was not affected. This is not surprising, since the phosphorylation of choline has been shown to be unaffected by moderate decreases in ATP levels in the myopathic heart [10]. Although a more severe drop in ATP levels would certainly curtail the formation of phosphocholine, it is clear from this study that phosphocholine formation was maintained even at 40% of the normal level of ATP.

Cardiac CTP was decreased to 60% of the normal value at 30 min of hypoxic perfusion, yet the conversion of phosphocholine to CDP-choline (and the synthesis of phosphatidylcholine) was not affected. There was also no change in the cytidylyltransferase activity (cytosolic and microsomal) under such conditions. This is indeed surprising, since a decrease in CTP to 66% of the normal level in the myopathic heart had a profound effect on the formation of CDP-choline and triggered the activation of the cytidylyltransferase [10]. One plausible explanation to the apparent discrepancy is that in cardiomyopathy the lowering of the CTP was instilled through a prolonged period of time. The prolonged decrease in CTP level might contribute to a significant decrease in the synthesis of CDP-choline and might thereby trigger the adaptive changes in cytidylyltransferase activity. Although the level of CTP was decreased after 30 min of hypoxic perfusion, such a drop occurred very rapidly and did not have an immediate effect on the synthesis of CDP-choline. Indeed, no change in CDP-choline labelling was observed at this time when compared with the control. Hence it is logical to surmise that no compensatory action of the cytidylyltransferase was required. When the level of CTP was further diminished (at 60 min of hypoxic perfusion), the rate of CDP-choline synthesis was significantly reduced. In order to compensate for the decrease in CDP-choline biosynthesis, cytidylyltransferase activity was enhanced by translocation of the enzyme to the microsomal fraction.

It is generally accepted that the microsomal form of cytidylyltransferase is the active form [32–34], and the translocation of the enzyme from the cytosolic to the microsomal form in some cells is regarded as a regulatory mechanism for the maintenance of CDP-choline, and subsequently phosphatidylcholine biosynthesis [33,34]. Based on the determination of the specific and total activities of the cytidylyltransferase in the subcellular fractions, it is clear that enzyme translocation took place in the hypoxic heart. The ability of the cytosol from the hypoxic heart to enhance enzyme translocation *in vitro* suggests that the factor for promoting translocation is located in the cytosol. At present, two mechanisms have been suggested for the translocation of the cytidylyltransferase: (1) enzyme phosphorylation/dephosphorylation [39] and (2) translocation induced by lipids [37]. Although direct evidence for enzyme phosphorylation/dephosphorylation is not well-established, enzyme translocation mediated by fatty acids has been demonstrated [37,40–43]. Hence, the higher fatty acid content in the hypoxic cytosol may be a key factor in causing the translocation of cytidylyltransferase to the microsomal compartment. The elevation of fatty acid level during hypoxia has been documented [12,44].

In spite of the decrease in radioactivity incorporated into phosphatidylcholine, there was no detectable change in phosphatidylcholine content in the hypoxic heart. It is possible that the rates of biosynthesis and catabolism of phosphatidylcholine in the heart are highly co-ordinated, and the decrease in its rate of synthesis might cause of corresponding decrease in its catabolism. Alternatively, there might be a fall in total phosphatidylcholine content in the heart which was not detectable at 60 min of hypoxic treatment but which would become more apparent if the heart was placed under longer periods of hypoxia. Another intriguing aspect is that the pool sizes of the choline-containing metabolites in the CDP-choline pathway were not changed during hypoxic treatment, despite the changes of radioactivities associated with these fractions. One explanation is that labelled choline taken up by the heart (and its subsequent metabolites) did not equilibrate with the endogenous pool of metabolites. Hence the accumulation of radioactivity in the phosphocholine fraction and the decrease of radioactivity in CDP-choline fraction did not affect the actual pool sizes of these metabolites in the hypoxic heart. The existence of separate choline pools in the hamster heart has been reported [5], and the channelling of labelled substrates in the CDP-choline pathway has been postulated [45].

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