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EVALUATION OF HUMAN BLOOD LYMPHOCYTES AS A MODEL TO STUDY THE EFFECTS OF DRUGS ON HUMAN MITOCHONDRIA

EFFECTS OF LOW CONCENTRATIONS OF AMIODARONE ON FATTY ACID OXIDATION, ATP LEVELS AND CELL SURVIVAL

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Abstract—Human lymphocytes were assessed as a cellular model for determining the effects of drugs on human mitochondria. Formation of total oxidized ^{14}C-products was maximal with 1 mM [U-^{14}C]-palmitic acid, was linear for 90 min, linear with the number of lymphocytes, and decreased by 95% and 77% in the presence of 30 μM rotenone and 2 mM KCN. Seven drugs were tested which had previously been shown to inhibit β-oxidation in animals; all decreased formation of total oxidized ^{14}C-products by human lymphocytes, but with different IC_{50} values: 35 μM with amiodarone, 2.75 mM with tetracycline and amineptine, 3.75 mM with tianeptine, and more than 10 mM for valproic acid and the ibuprofen enantiomers. Formation of [^{14}C]CO_2 either increased or decreased, in relation to the various effects of these drugs on coupling, β-oxidation, and the tricarboxylic acid cycle. There was a general trend for some relationship between inhibition of fatty acid oxidation and loss of cellular ATP. Those compounds, however, which uncoupled oxidative phosphorylation (2,4-dinitrophenol, amiodarone, ibuprofen) and/or inhibited the mitochondrial respiratory chain (amiodarone, rotenone, KCN) resulted in comparatively higher ATP depletion. Amiodarone, a drug which produces several effects (uncoupling, inhibition of β-oxidation, of the tricarboxylic acid cycle and of the respiratory chain), caused a dramatic decrease in cellular ATP and cell viability at low concentrations (20–100 μM). Both these effects were prevented by the addition of 5 mM glucose, a substrate for anaerobic glycolysis. We conclude that human lymphocytes may be a useful model for assessing the effects of drugs on human mitochondrial function. IC_{50} values determined with this model may not necessarily apply, however, to other cells.

Many drugs have been shown to inhibit directly mitochondrial fatty acid β-oxidation in rats and mice, including valproic acid [1–3], tetracycline [4], several tetracycline derivatives [5], salicylic acid [6–8], amineptine [9], tianeptine [10], amiodarone [11, 12], and several 2-arylpropionic antiinflammatory drugs, such as pirprofen [13], tiaprofenic acid [13], both *R*-(−) and *S*-(+) ibuprofen enantiomers [14, 15] and flurbiprofen [15]. In addition, chemicals which inhibit the mitochondrial respiratory chain, like rotenone [16], antimycin [17], KCN [18] and amiodarone [12] may also lead to upstream inhibition of mitochondrial fatty acid β-oxidation. Such effects may have serious toxicological consequences. Inhibition of the mitochondrial respiratory chain and/or β-oxidation decreases the energy supply of the cell, and may lead to ATP depletion and cell death. Furthermore, inhibition of β-oxidation leads to increased esterification of fatty acids into triglycerides, which are deposited in the form of small lipid vesicles [19]. Microvesicular steatosis is a severe liver disease, which may lead to coma and death [19, 20].

It is noteworthy, however, that all the above mentioned studies were performed on liver mitochondria from rats or mice. Since both nuclear and mitochondrial genes coding for β-oxidation enzymes and respiratory chain polypeptides are somewhat divergent among rats, mice and humans [21, 22], it is important to assess the effects of drugs in a human system. While human hepatocytes would obviously be the best model, large human liver samples are increasingly difficult to acquire due to the generalization of liver transplantation, and must now await relatively uncommon surgical interventions, such as partial hepatectomies for localized hepatic tumors. In contrast, human blood lymphocytes are easily obtained.

The goal of the present study was to assess the potential usefulness of human lymphocytes in determining the effect of drugs and other chemicals on fatty acid β-oxidation in human mitochondria. For this purpose, the characteristics for the assay of the oxidation of [U-^{14}C]palmitic acid by human lymphocytes were first determined, and then the effects of drugs and chemicals (known to exhibit various effects on mitochondrial function in animals) on [U-^{14}C]palmitic acid oxidation, cellular ATP, and cell viability in human lymphocytes were investigated.

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MATERIALS AND METHODS

Materials. [U-^{14}C]palmitic acid (800 mCi/mmol)

was purchased from New England Nuclear (Boston, MA, U.S.A.). The lymphocyte separation medium (MSL) containing Ficoll and diatrizoate was from Eurobio (Les Ulis, France).

Amiodarone hydrochloride and *N*-desethylamiodarone hydrochloride were gifts from Clin-Midy (Montpellier, France). The sodium salt of tianeptine, and amineptine hydrochloride were donated by the Institut de Recherches Internationales Servier (Courbevoie, France). The *S*-(+) and *R*-(−) enantiomers of ibuprofen were gifts from the Boots Company plc (Nottingham, U.K.). Optical purity was 99.6% for the *S*-(+) enantiomer and 99.4% for the *R*-(−) enantiomer.

2-Methoxyethanol was from Prolabo (Paris, France). All other drugs or chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.), Merck (Darmstadt, Germany), or Boehringer (Mannheim, Germany).

Compounds were dissolved either in water (sodium salt of tianeptine, amineptine hydrochloride, tetracycline hydrochloride, sodium valproate, KCN), or in 0.1 N NaOH (ibuprofen enantiomers) or in ethanol [amiodarone, *N*-desethylamiodarone, rotenone, 2,4-dinitrophenol (DNP*)]. Aqueous solutions were neutralized before use. When compounds were dissolved in ethanol, only a small volume of ethanol (2–5 μL) was added in the test tubes, with the same volume added to control tubes.

Subjects. Male and female healthy volunteers from the medical and research staff (age 25–40 years) kindly gave informed consent to have a 40-mL venous blood sample collected into sterile tubes containing lithium heparin.

Isolation of human lymphocytes. Lymphocytes were prepared using an MSL–Ficoll gradient, as recommended by the manufacturer with minor modifications. Blood was diluted with an equal volume of 0.9% w/v saline solution and layered onto the lymphocyte separation medium. After centrifugation at 400 *g* for 20 min at 20°, cells were removed from the plasma–Ficoll interface and washed twice with Krebs–Ringer-bicarbonate buffer (KRB buffer) containing (in millimolar) KH_2PO_4, 1.2; $CaCl_2$, 1; $MgSO_4$, 1.2; KCl, 4.8; $NaHCO_3$, 24; NaCl, 120 (pH 7.35–7.45) and 1% defatted bovine serum albumin. After the last centrifugation (300 *g* for 10 min) lymphocytes were resuspended in the same KRB buffer. The overall yield from 40 mL of blood ranged from 50 to 80 × 10^6 cells, with 95% of the isolated cells being lymphocytes. At this step, lymphocyte viability was at least 99%.

[U-^{14}C]*Palmitic acid oxidation in human lymphocytes.* Oxidation of [U-^{14}C]palmitic acid by human lymphocytes was assessed by measuring both (a) the formation of acid-soluble β-oxidation ^{14}C-products, representing mainly ketone bodies and, to a small extent, tricarboxylic acid cycle intermediates [23–25] and (b) the formation of [^{14}C]CO_2 generated by the tricarboxylic acid cycle. Preliminary experiments were performed to determine the characteristics of the assay. The formation rate of ^{14}C-products (acid-soluble β-oxidation products, and [^{14}C]CO_2) was studied (a) with various concentrations of palmitic acid, (b) after various incubation times, (c) with a variable number of lymphocytes, (d) with or without L-carnitine (2 mM), and (e) with two sorts of bovine serum albumin, either an essentially fat-free albumin, or a charcoal-treated dialysed albumin which is a defatted albumin [26].

From these preliminary experiments, the following standard protocol was used. Lymphocytes resuspended in the KRB buffer (3–6 × 10^6 cells) were preincubated for 5 min at 37°. The studied compound was then added to the test tubes, and the incubation mixture was brought to 2 mL by adding 400 μL of a saline solution containing [U-^{14}C]palmitic acid (final concentration, 1 mM; 0.20 μCi/2 mL) and defatted bovine serum albumin (final concentration in the assay, 23 mg/2 mL or 1.15%). The tubes were then tightly capped with a plastic stopper pierced by two needles, and swept continuously by an air flow which was bubbled into 10 mL of ethanolamine/2-methoxyethanol (30/70, v/v) to trap [^{14}C]CO_2 as described previously [8, 10]. The tubes were then incubated with slow shaking for 90 min at 37°. The reaction was stopped by injecting 0.4 mL of cold citric acid (2 M, pH 1.5) into the incubation mixture. Trapping of [^{14}C]CO_2 was continued for an additional 30 min. A 3 mL aliquot of ethanolamine/2-methoxyethanol was counted for [^{14}C]CO_2, and the incubation tubes were then placed in ice, and the following compounds were added: 6 μmol unlabeled palmitic acid dissolved in 100 μL ethanol and 0.6 mL of 6 M perchloric acid. Unlabeled palmitic acid acts as an insoluble carrier for any remaining [^{14}C]fatty acids [27, 28]. After 1 hr, the tubes were centrifuged at 3000 *g* for 20 min at 4°, and the supernatant was filtered through a Whatman paper (folded filter paper 2V). Acid-soluble β-oxidation ^{14}C-products were then counted in an aliquot of the filtered supernatant.

From the known specific radioactivity of [U-^{14}C]-palmitate, and the expected specific radioactivity of derived [^{14}C]ketone bodies (usually, 1/8 of that of palmitic acid) or derived [^{14}C]CO_2 (1/16 of that of palmitic acid), results were expressed as nmol of acid-soluble β-oxidation ^{14}C-products, and nmol of [^{14}C]CO_2, formed per hour and per 10^6 viable lymphocytes, while the sum of these two values was termed total oxidized ^{14}C-products. Results had to be expressed per viable lymphocytes since some compounds, particularly amiodarone, induced loss of viability during the 90 min of incubation. After verifying that the decrease in cell viability was linear with time (see Results), the mean number of lymphocytes active during the 90 min experiment could be calculated as the arithmetic mean of the number of lymphocytes present at zero time and the number of lymphocytes still alive at the end of the experiment (90 min).

Cytotoxicity. Viability of human lymphocytes was determined in parallel incubations performed as described above, but with unlabeled palmitic acid. After different incubation times, an aliquot of the cell suspension was mixed with 0.2% Trypan blue, and 150 cells were examined for Trypan blue exclusion.

* Abbreviations: KRB, Krebs–Ringer-bicarbonate buffer; IC_{50}, concentration of drug decreasing the activity by 50%; DNP, 2,4-dinitrophenol; VPA, valproic acid.

In some experiments, human lymphocytes were preincubated for 1 hr with or without 5 mM glucose (which produces ATP through glycolysis), and with or without 0.5 mM iodoacetate, a glycolysis inhibitor [29], and were further incubated for 90 min with or without 100 μM amiodarone. Cell viability was then measured. Glucose was used as a glycolysis substrate rather than fructose because lymphocytes do not utilize fructose [30].

ATP. Lymphocytes ($1–2 \times 10^6$ cells) were incubated at 37° in 400 μL KRB buffer (pH 7.35–7.45) containing 1 mM unlabeled palmitic acid and 1.15% defatted bovine serum albumin, with or without the studied compound. Incubation was stopped at different times by adding the same volume of ice-cold 1 N perchloric acid. The samples were placed in ice, and centrifuged at 2000 *g* for 15 min, at 4°. Aliquots (400 μL) of the supernatants were neutralized with 45 μL of 5 M K_2CO_3, and centrifuged again at 2000 *g* for 15 min, at 4°. An aliquot of the supernatant was diluted in ice-cold freshly distilled water, and ATP was measured by a luciferin–luciferase assay [31], as described by Stanley and Williams [32]. The luminescent signal (measured with a Packard Tri-Carb liquid scintillation counter) was found to be proportional to the ATP content in a concentration range of 2.5×10^{-10} to 2.5×10^{-8} M (final concentration in the assay). Results were expressed as nmol per 10^6 viable lymphocytes.

In some experiments, human lymphocytes were preincubated for 1 hr with or without 5 mM glucose, and with or without 0.5 mM iodoacetate, and further incubated for 30 min with or without 100 μM amiodarone, before measurement of ATP levels.

Statistical analysis. The Student's *t*-test for independent data, or the Dunnett's *t*-test was used to assess the significance of differences between means, as appropriate.

RESULTS

Characteristics of [U-^{14}C]*palmitic acid oxidation by human lymphocytes*

Several preliminary experiments were performed to determine the characteristics of [U-^{14}C]palmitic acid oxidation by human lymphocytes, and to select a standard protocol. Incubations performed for 90 min with various concentrations of [U-^{14}C]palmitic acid (and 1.15% of defatted bovine serum albumin) showed Michaelis–Menten kinetics (Fig. 1 and data not shown). A saturating concentration of 1 mM (Fig. 1) was selected for additional studies. At this concentration, formation of acid-soluble β-oxidation ^{14}C-products, and formation of [^{14}C]CO_2, represented about 75 and 25%, respectively, of total oxidation products (Fig. 1). Therefore both formation of acid-soluble β-oxidation ^{14}C-products, and formation of [^{14}C]CO_2 were routinely assessed in the standard protocol.

Time course studies, performed with 1 mM [U-^{14}C]palmitic acid and 1.15% defatted bovine serum albumin, showed that the increase in acid-soluble β-oxidation [^{14}C]products tended to even out after 90 min, while the production of [^{14}C]CO_2 possibly showed an early lag phase (Fig. 1). Formation of total oxidized products was grossly linear with time up to 120 min (Fig. 1). An incubation time of 90 min was selected for further studies.

Experiments performed with various numbers of human lymphocytes, incubated with 1 mM [U-^{14}C]-palmitic acid and 1.15% defatted bovine serum albumin, showed that the reaction rate (nmol/hr) increased linearly with the number of lymphocytes, in a range from 2 to 9×10^6 cells (Fig. 1). A lymphocyte number ranging from 3 to 6×10^6 cells was therefore used in the standard protocol.

Incubations performed with two types of albumin (1.15%), and 1 mM [U-^{14}C]palmitic acid for 90 min, showed that the formation of total oxidized ^{14}C-products (mean ± SE for four determinations) was essentially the same with fatty acid-free bovine serum albumin and charcoal-treated dialysed (defatted) bovine serum albumin (7.5 ± 0.5 and 7.6 ± 0.8 nmol/hr/10^6 lymphocytes, respectively). The less expensive, defatted preparation was used in the standard protocol.

Addition of exogenous L-carnitine (0.5–1.3 mM) has been shown to improve the oxidation of palmitic acid by rat hepatocytes [33, 34]. With human lymphocytes, however, addition of 2 mM L-carnitine to the incubation medium did not increase the formation rate of total oxidized ^{14}C-products. The rate was 9.1 ± 0.6 nmol/hr/10^6 lymphocytes without carnitine and 9.3 ± 0.5 when 2 mM L-carnitine was added to the incubation medium (mean ± SE for five determinations). Therefore carnitine was not added in the standard protocol.

With this standard protocol, human lymphocytes from different persons were found to produce between 5 and 12 nmol of total oxidized ^{14}C-products/hr/10^6 lymphocytes.

The effects of two mitochondrial poisons on the oxidation of [U-^{14}C]palmitic acid by human lymphocytes were also studied (Fig. 2). Rotenone and KCN respectively inhibit electron flow at the level of complex I and IV of the respiratory chain [35]. Rotenone (30 μM) and KCN (2 mM) markedly decreased the formation of both [^{14}C]CO_2 and acid-soluble β-oxidation ^{14}C-products in human lymphocytes (Fig. 2). Formation of total oxidized ^{14}C-products was decreased by 95 and 77%, respectively, in the presence of 30 μM rotenone and 2 mM KCN (Fig. 2). These observations, together with recent results of Wanders and Ijlst [36], suggest that the oxidation of [U-^{14}C]palmitic acid is mainly mediated by mitochondria in human lymphocytes (see Discussion).

Effects of drugs on [U-^{14}C]*palmitic acid oxidation by human lymphocytes*

Using the standard protocol described above, the effects of seven drugs, namely *S*-(+) ibuprofen, *R*-(−) ibuprofen, valproic acid (VPA), tianeptine, amineptine, tetracycline and amiodarone, were assessed on [U-^{14}C]palmitic acid oxidation by human lymphocytes (Figs 3 and 4, Table 1). These drugs have been previously shown to inhibit the mitochondrial oxidation of palmitic acid in rat or mouse mitochondria, or in rat hepatocytes (Table 1).

S-(+) Ibuprofen, *R*-(−) ibuprofen and VPA were weak inhibitors of [U-^{14}C]palmitic acid oxidation by

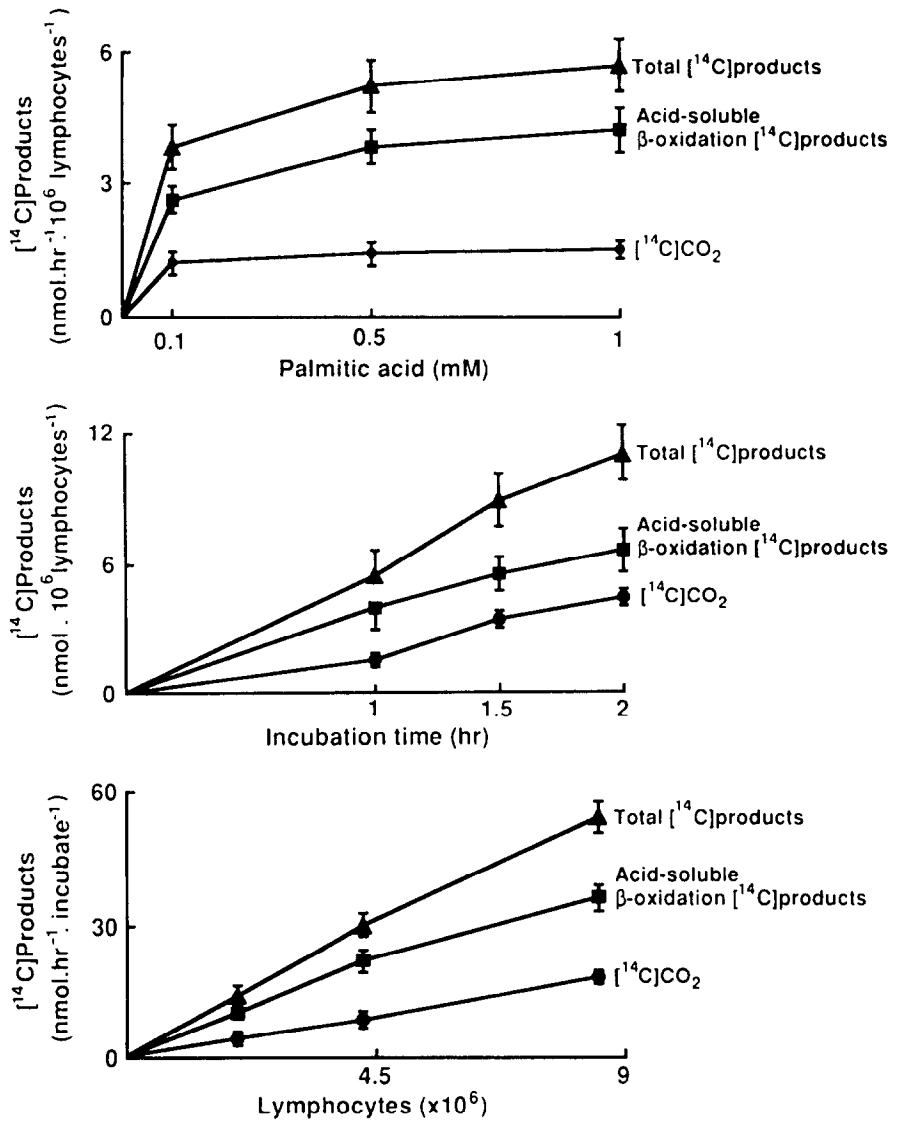

Fig. 1. Oxidation of [U-^{14}C]palmitic acid by human lymphocytes under various experimental conditions. The formation of acid-soluble β-oxidation ^{14}C-products, [^{14}C]CO_2, and their sum, termed total oxidized [^{14}C]products, was determined under various conditions. In a first experiment, lymphocytes (4–8 × 10^6 cells) were incubated for 90 min at 37° with 0.1, 0.5 or 1 mM [U-^{14}C]palmitic acid and 1.15% defatted bovine serum albumin. In a second experiment, lymphocytes (3–7 × 10^6 cells) were incubated with 1 mM [U-^{14}C]palmitic acid and 1.15% defatted bovine serum albumin for 60, 90 or 120 min. In the last experiment, a variable number of lymphocytes was incubated for 90 min with 1 mM [U-^{14}C]-palmitic acid and 1.15% defatted bovine serum albumin. Results are means ± SE for four to six determinations.

human lymphocytes (Fig. 3). Interestingly, these drugs had different effects on (a) the formation of acid-soluble β-oxidation [^{14}C]products and (b) the formation of [^{14}C]CO_2 (Fig. 3). Indeed, *S*-(+) ibuprofen, *R*-(−) ibuprofen and VPA, at concentrations of 10 mM, decreased the formation of acid-soluble β-oxidation ^{14}C-products by 47, 41 and 32%, respectively (Fig. 3). In contrast, they did not decrease, and even tended to increase (non significantly) the production of [^{14}C]CO_2 (Fig. 3). As a consequence of these contrasting effects, *S*-(+) ibuprofen, *R*-(−) ibuprofen and VPA, at a concentration of 10 mM, elicited only moderate effects on the formation rate of total oxidized ^{14}C-products, this rate being decreased by 31, 25 and 19%, respectively (Fig. 3).

Tianeptine, amineptine and tetracycline decreased both the formation of acid-soluble β-oxidation ^{14}C-products and the formation of [^{14}C]CO_2 (Fig. 4). At a concentration of 2 mM, tianeptine, amineptine and tetracycline began to decrease the formation of total oxidized ^{14}C-products by 26, 22, and 30%,

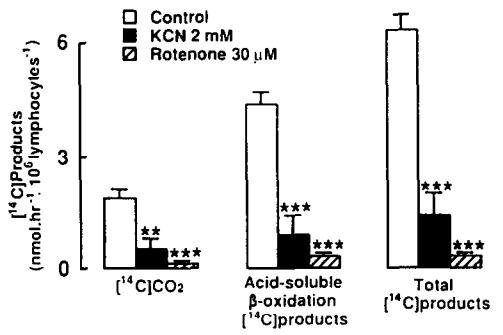

Fig. 2. Effects of KCN and rotenone on the formation of acid-soluble β-oxidation ^{14}C-products, $[^{14}C]CO_2$ and total oxidized ^{14}C-products from [U-^{14}C]palmitic acid by human lymphocytes. Lymphocytes were incubated for 90 min at 37° with 1 mM [U-^{14}C]palmitic acid and 1.15% defatted bovine serum albumin in the presence or absence of 2 mM KCN or 30 μM rotenone. Results are means ± SE for four to six determinations. Asterisks indicate significant differences from incubations performed without mitochondrial inhibitors (**$P < 0.01$, ***$P < 0.001$).

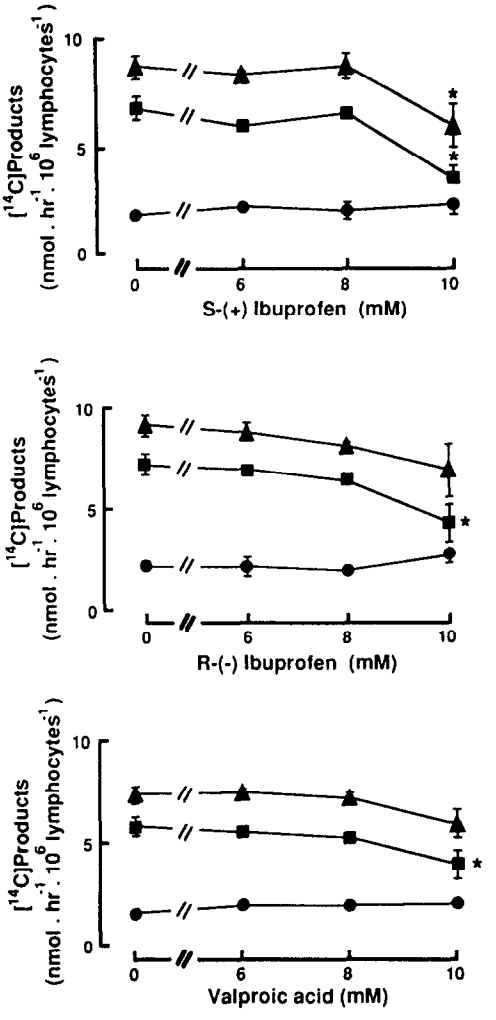

Fig. 3. Effects of various concentrations of *R*-(−) ibuprofen, *S*-(+) ibuprofen or VPA on the formation of acid-soluble β-oxidation ^{14}C-products, $[^{14}C]CO_2$ and total oxidized ^{14}C-products from [U-^{14}C]palmitic acid by human lymphocytes. Lymphocytes were incubated for 90 min at 37° with 1 mM [U-^{14}C]palmitic acid and 1.15% defatted bovine serum albumin, with or without the studied drug. Results are means ± SE for 4–12 determinations. (▲) Total ^{14}C-products, (■) acid-soluble β-oxidation ^{14}C-products, (●) $[^{14}C]CO_2$. Asterisks indicate significant differences from control incubations (*$P < 0.05$).

respectively (Fig. 4). The estimated IC_{50} (the concentration of drug decreasing the activity by 50%) for these products was 3.75, 2.75 and 2.75 mM, respectively (Table 1).

In its protonated form, amiodarone has been shown to enter the mitochondrion along the mitochondrial membrane potential [12]. Inside the mitochondrial matrix, where the pH is more alkaline, protonated amiodarone dissociates into a proton and uncharged amiodarone [12]. At extremely low concentrations of amiodarone, the entry of protons leads to an uncoupling effect in isolated mouse liver mitochondria [12]. At slightly higher concentrations, however, the accumulation of amiodarone in the mitochondria inhibits both the respiratory chain and the β-oxidation process [11, 12]. In the present study, amiodarone was by far the most potent inhibitor of [U-^{14}C]palmitic acid oxidation by human lymphocytes (Fig. 4). Indeed, at concentrations of 30, 40, 50 and 100 μM, this substance inhibited the formation of total oxidized ^{14}C-products by 36, 64, 80 and 89%, respectively (Fig. 4), with an IC_{50} of approximately 35 μM (Table 1). At these concentrations, both the formation of acid-soluble β-oxidation ^{14}C-products and the formation of $[^{14}C]CO_2$ were inhibited (Fig. 4). At extremely low concentrations (20 μM), however, the formation of acid-soluble β-oxidation ^{14}C-products was not inhibited, and the formation of $[^{14}C]CO_2$ was significantly increased (Fig. 5). Similar effects were seen with the prototypical uncoupler of oxidative phosphorylation, DNP (160 μM), which did not change the formation of acid-soluble β-oxidation ^{14}C-products, but increased the formation of $[^{14}C]CO_2$ (Fig. 5).

Amiodarone is demethylated into *N*-desethylamiodarone, its main metabolite by cytochrome P450 [37]. In isolated mouse liver mitochondria, the inhibitory effect of *N*-desethylamiodarone on [U-^{14}C]palmitic acid oxidation was similar to that of unchanged amiodarone [11]. In the present study, *N*-desethylamiodarone was also shown to inhibit markedly the oxidation of [U-^{14}C]palmitic acid by human lymphocytes. Indeed, the formation of total oxidized ^{14}C-products was 6.2 ± 0.2 nmol/hr/10^6 lymphocytes in control cells, but only 0.6 ± 0.2 in the presence of 100 μM *N*-desethylamiodarone (mean ± SE for three to four determinations).

Cellular ATP and cytotoxicity

Table 2 shows the effects of the seven drugs studied, as well as those of DNP, KCN and rotenone (each compound was added at the highest concentration used in the above-mentioned

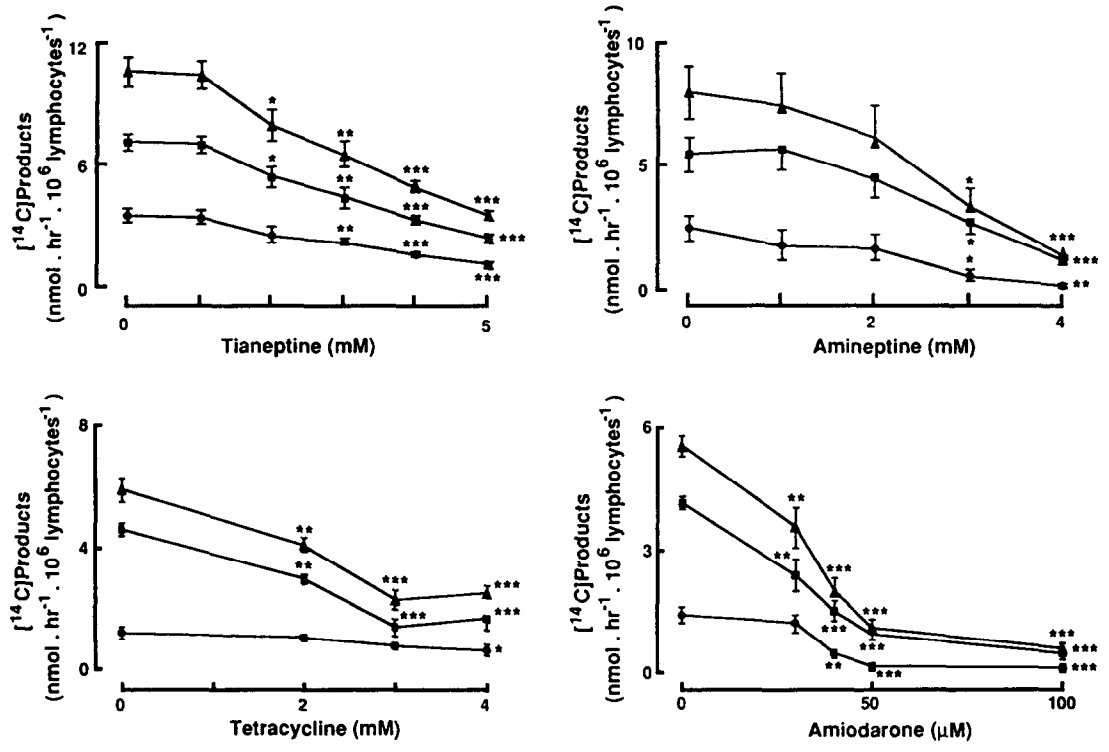

Fig. 4. Effects of various concentrations of tianeptine, amineptine, tetracycline, or amiodarone on the formation of acid-soluble β-oxidation ^{14}C-products, $[^{14}C]CO_2$ and total oxidized ^{14}C-products from [U-^{14}C]palmitic acid by human lymphocytes. Lymphocytes were incubated for 90 min at 37° with 1 mM [U-^{14}C]palmitic acid and 1.15% defatted bovine serum albumin in the absence or presence of the studied drug. Results are means ± SE for 4–12 determinations. (▲) Total ^{14}C-products, (■) acid-soluble β-oxidation ^{14}C-products, (●) $[^{14}C]CO_2$. Asterisks indicate significant differences from control incubations (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Table 1. IC_{50} for the oxidation of palmitic acid by human lymphocytes in the present study, as compared to IC_{50} values obtained in isolated mouse or rat liver mitochondria, or isolated rat hepatocytes in previous studies

Drug	IC_{50} for formation of total oxidized ^{14}C-products from [U-^{14}C]palmitic acid by human lymphocytes in the present study (mM)	IC_{50} for the oxidation of palmitic acid by mouse or rat liver mitochondria or rat hepatocytes in previous studies (mM)
S-(+) Ibuprofen	>10	1.45* [14]
R-(−) Ibuprofen	>10	>2* [14]
VPA	>10	0.125† [2]
		2.5‡ [1]
Tianeptine	3.75	0.65* [10]
Amineptine	2.75	1.25* [9]
Tetracycline	2.75	0.85* [4]
Amiodarone	0.035	0.10* [11]

IC_{50} values in human lymphocytes were estimated from values reported in Figs 3 and 4.

* Formation of acid-soluble β-oxidation ^{14}C-products from [U-^{14}C]palmitic acid was measured in isolated mouse liver mitochondria.

† Oxidation of palmitoyl-carnitine was assessed by polarographic measurement of oxygen consumption in isolated rat liver mitochondria.

‡ Formation of acid-soluble β-oxidation ^{14}C-products from [1-^{14}C]palmitic acid was measured in isolated rat hepatocytes.

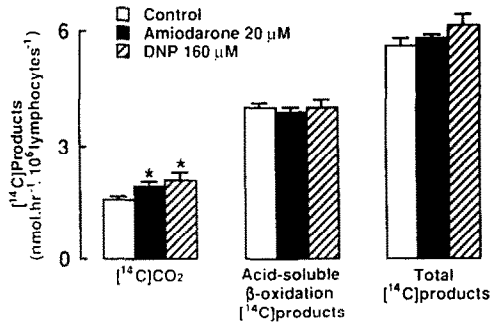

Fig. 5. Effects of DNP or a very low concentration of amiodarone on the production of acid-soluble β-oxidation ^{14}C-products, [^{14}C]CO_2 and total oxidized ^{14}C-products from [U-^{14}C]palmitic acid by human lymphocytes. Lymphocytes were incubated for 90 min at 37° with 1 mM [U-^{14}C]palmitic acid and 1.15% defatted bovine serum albumin in the presence or absence of 160 μM DNP or 20 μM amiodarone. Results are means ± SE for six to eight determinations. Asterisks indicate significant differences from control incubations (*$P < 0.05$).

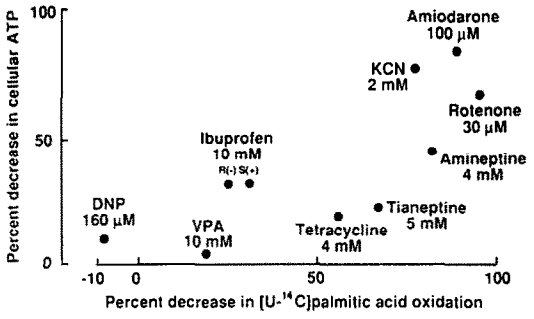

Fig. 6. Relationship between the decrease in the formation of total oxidized ^{14}C-products from [U-^{14}C]palmitic acid and the decrease in cellular ATP in human lymphocytes. The plot has been drawn from results reported in Figs 3 and 4 and in Table 2.

experiments) on cellular ATP levels after 30 min of incubation with 1 mM palmitic acid and 1.15% defatted bovine serum albumin. Figure 6 shows the relationship between (a) the percentage of decrease in the formation of total oxidized ^{14}C-products from [U-^{14}C]palmitic acid, and (b) the percentage of decrease in cellular ATP with the 10 compounds. There was a general tendency for ATP to decrease as the oxidation of palmitic acid decreased (Fig. 6), a finding which supports the view that mitochondrial oxidation of fatty acids is an important source of energy in the present *in vitro* model (where incubations were performed without glucose). Six compounds, however, namely DNP, *R*-(−), ibuprofen, *S*-(+) ibuprofen, KCN, rotenone and amiodarone, resulted in an ATP depletion that was somewhat higher than that produced by other compounds producing comparable effects on [U-^{14}C]palmitic acid oxidation (Fig. 6). Interestingly (see Discussion), these six compounds are either uncouplers of oxidative phosphorylation (DNP, ibuprofen enantiomers and amiodarone) and/or inhibitors of the respiratory chain (amiodarone, rotenone and KCN). Both effects should lead to further impairment of ATP formation.

The most dramatic decrease in cellular ATP levels was observed with 100 μM amiodarone (Fig. 6), a drug which produces four effects on mitochondrial function (uncoupling, inhibition of β-oxidation, inhibition of the tricarboxylic acid cycle and inhibition of the respiratory chain) [11, 12]. Indeed, comparison of the time course for ATP depletion (Fig. 7) showed that the rate of depletion in the first 15 min was about twice as high with 100 μM amiodarone as with 30 μM rotenone.

Table 2. Cellular ATP levels and percentage of dead lymphocytes after incubation with various chemicals

	Cellular ATP at 30 min			Percentage of dead lymphocytes at 90 min	
	Control (nmol/10^6 lymphocytes)	Treated	Per cent decrease (%)	Control (%)	Treated (%)
DNP (0.16 mM)	4.7 ± 0.1	4.2 ± 0.1*	11	1 ± 1	2 ± 1
S-(+) Ibuprofen (10 mM)	4.8 ± 0.1	3.2 ± 0.1†	33	1 ± 1	4 ± 1
R-(−) Ibuprofen (10 mM)	4.8 ± 0.1	3.2 ± 0.1†	33	1 ± 1	4 ± 1
VPA (10 mM)	4.5 ± 0.2	4.3 ± 0.2	4	2 ± 1	2 ± 1
Tianeptine (5 mM)	4.5 ± 0.2	3.5 ± 0.2†	22	2 ± 1	11 ± 1*
Amineptine (4 mM)	4.4 ± 0.2	2.5 ± 0.1†	44	3 ± 1	12 ± 4*
Tetracycline (4 mM)	4.8 ± 0.1	3.9 ± 0.1†	19	2 ± 1	10 ± 2*
Amiodarone (0.1 mM)	4.0 ± 0.1	0.7 ± 0.1†	83	1 ± 1	60 ± 7†
KCN (2 mM)	4.7 ± 0.1	1.0 ± 0.1†	79	2 ± 1	3 ± 1
Rotenone (0.03 mM)	4.0 ± 0.1	1.3 ± 0.2†	68	1 ± 1	2 ± 1

Human lymphocytes were incubated at 37° for 30 or 90 min with 1 mM palmitic acid and 1.15% defatted bovine serum albumin.

Results are means ± SE for 4–11 determinations.

Significantly different from control incubations, *$P < 0.05$, †$P < 0.01$.

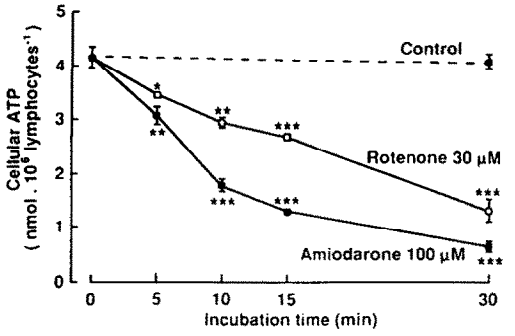

Fig. 7. Time course for the decrease in ATP in control human lymphocytes and in lymphocytes incubated with rotenone or amiodarone. Lymphocytes were incubated at 37° for 5, 10, 15 or 30 min with 1 mM unlabeled palmitic acid and 1.15% defatted bovine serum albumin in the presence or absence of 30 μM rotenone or 100 μM amiodarone. Results are means ± SE for three to eight determinations. Asterisks indicate significant differences from control incubations (*P < 0.05, **P < 0.01, ***P < 0.001).

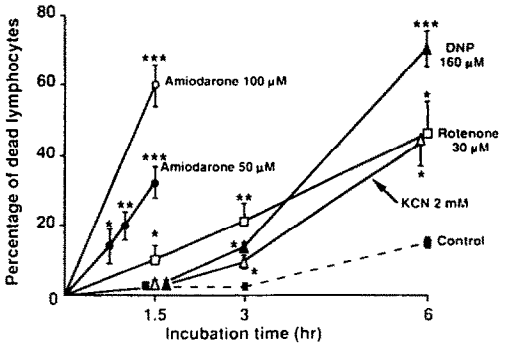

Fig. 9. Time course for cytotoxicity in control human lymphocytes or lymphocytes incubated with amiodarone, KCN, rotenone or DNP. Lymphocytes were incubated at 37° for 45, 60 or 90 min (with amiodarone), or 1.5, 3 or 6 hr (with KCN, rotenone or DNP) with 1 mM unlabeled palmitic acid and 1.15% defatted bovine serum albumin in the presence or absence of 50 or 100 μM amiodarone, 2 mM KCN, 30 μM rotenone or 160 μM DNP. Results are means ± SE for 3–11 determinations. Asterisks indicate significant differences from control incubations (*P < 0.05, **P < 0.01, ***P < 0.001).

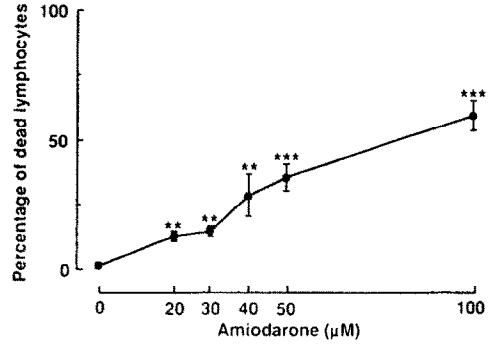

Fig. 8. Cytotoxicity of various concentrations of amiodarone in human lymphocytes. Lymphocytes were incubated for 90 min at 37° with 1 mM unlabeled palmitic acid and 1.15% defatted bovine serum albumin with or without amiodarone (20–100 μM). Results are means ± SE for 4–11 determinations. Asterisks indicate significant differences from control incubations (**P < 0.01, ***P < 0.001).

Table 2 also shows the percentage of dead lymphocytes after 90 min of incubation with the 10 compounds (each at the highest concentration studied). Cytotoxicity reached 60% with 100 μM amiodarone, but remained low (12% or less) with the nine other compounds (Table 2). Figure 8 shows the effects of various concentrations of amiodarone on the percentage of dead lymphocytes after 90 min of incubation. Significant lymphocytotoxicity (13%) was already apparent with 20 μM of amiodarone, and increased nearly linearly with increasing concentrations (Fig. 8).

Figure 9 compares the time course of lymphocytotoxicity with two concentrations of amiodarone (50 and 100 μM) and with 160 μM DNP, 30 μM rotenone and 2 mM KCN. The percentage of dead lymphocytes increased linearly with time with 50 μM amiodarone reaching 32% at 90 min, and reaching 60% at 90 min with 100 μM of this substance (Fig. 9). Lymphocytotoxicity occurred much more slowly with DNP, rotenone and KCN, reaching 70, 46 and 45%, respectively, after 6 hr of incubation (Fig. 9).

We tried to prevent the high cytotoxicity of amiodarone by using glucose, a substrate which may produce ATP directly through glycolysis. Human lymphocytes were preincubated for 60 min with or without 5 mM glucose, and with or without 0.5 mM iodoacetate (a glycolysis inhibitor), and then incubated for another 30 or 90 min with or without 100 μM amiodarone (Table 3).

In lymphocytes not exposed to amiodarone, the addition of either glucose alone or iodoacetate alone did not modify basal ATP values (Table 3). However, the addition of both glucose and iodoacetate decreased basal ATP values presumably because under these conditions glucose used two ATP molecules in the formation of glucose 6-phosphate and fructose 1,6-diphosphate, while glycolysis was inhibited by iodoacetate (at the level of glyceraldehyde 3-phosphate dehydrogenase) thus preventing the formation of the four ATP molecules normally obtained in the conversion to lactate, and resulting in a net loss of ATP (Table 3). Cell viability remained high in the lymphocytes incubated without amiodarone (Table 3).

In lymphocytes exposed to amiodarone, the addition of glucose alone partially prevented both the amiodarone-induced loss of cellular ATP, and the loss of cell viability (Table 3). Co-incubation of

Table 3. Effects of glucose (a glycolysis substrate), without or with iodoacetate (a glycolysis inhibitor) on cellular ATP and the percentage of dead lymphocytes after incubation with 100 μM amiodarone

Incubation system	Cellular ATP at 30 min			Percentage of dead lymphocytes at 90 min	
	Control (nmol/10^6 lymphocytes)	Amiodarone	Per cent decrease (%)	Control (%)	Amiodarone (%)
Standard system	3.3 ± 0.2	1.2 ± 0.2*	64	3 ± 1	67 ± 9*
With 5 mM glucose	3.4 ± 0.2	2.2 ± 0.4*†	35	2 ± 1	34 ± 5*†
With 0.5 mM iodoacetate	3.0 ± 0.2	0.7 ± 0.1*	77	5 ± 2	74 ± 3*
With both 5 mM glucose and 0.5 mM iodoacetate	1.8 ± 0.1‡	0.9 ± 0.1*	50	6 ± 2	79 ± 2*

Human lymphocytes were preincubated at 37° for 60 min with 1 mM palmitic acid, 1.15% defatted bovine serum albumin, with or without 5 mM glucose, and with or without 0.5 mM iodoacetate, and then incubated for 30 or 90 min in the presence or absence of 100 μM amiodarone. Results are means ± SE for four to seven determinations.

* Significantly different from control incubations performed in the same incubation system, but without amiodarone, $P < 0.05$.

† Significantly different from incubations performed with amiodarone, but with the standard system, $P < 0.05$.

‡ Significantly different from control incubations performed with the standard system, $P < 0.05$.

iodoacetate and glucose prevented the beneficial effects of glucose against amiodarone-induced ATP depletion and loss in cell viability (Table 3); the addition of iodoacetate alone did not significantly modify ATP values and cell viability in amiodarone-exposed lymphocytes (Table 3).

As glucose only partially reversed the cytotoxicity of amiodarone (Table 3), the lymphocytotoxicity of this anti-arrhythmic agent may involve effects besides those on mitochondria. To investigate this point, the cytotoxicity produced by 100 μM amiodarone was compared with that produced by a combination of 160 μM DNP (uncoupling), 30 μM rotenone (inhibition of the respiratory chain) and 4 mM tetracycline (inhibition of β-oxidation and inhibition of the tricarboxylic acid cycle). This combination should reproduce the various effects of amiodarone on mitochondrial function. However, the 60% loss in viability observed after 90 min of incubation with amiodarone was not reproduced by the combination of the three compounds, and resulted in only 5% mortality (data not shown).

DISCUSSION

Mouse or rat liver mitochondria, or rat hepatocytes, have been previously used to assess the effects of drugs on the β-oxidation of fatty acids. To determine whether these drugs also inhibit β-oxidation in human mitochondria, human lymphocytes were studied as an alternative model.

Human lymphocytes were found to produce between 5 and 12 nmol of total oxidized ^{14}C-products/hr/10^6 lymphocytes in the presence of 1 mM [U-^{14}C]palmitic acid (Fig. 1). This concentration insured maximal oxidation rates (Fig. 1), and is in the physiological range, since plasma fatty acid concentrations range from 0.5 to 2 mM in humans [30]. The production of [^{14}C]CO_2 represented about 25% of the total oxidized products in human lymphocytes (Fig. 1). In contrast, [^{14}C]CO_2 production from [U-^{14}C]palmitic acid represented only 10% or less of the total oxidized products in hepatocytes [28, 33].

Rotenone (30 μM) and KCN (2 mM), two substances which lead to upstream inhibition of the mitochondrial β-oxidation of fatty acids [12, 16, 18], markedly decreased palmitic acid oxidation by human lymphocytes (Fig. 2). Likewise, 2-[5-(4-chlorophenyl)-pentyl]-oxirane-2-carboxylate, a powerful and specific inhibitor of mitochondrial carnitine palmitoyl-CoA transferase I, markedly inhibited the oxidation of [1-^{14}C]palmitic acid in human leukocytes [36]. Thus, the peroxisomal β-oxidation of palmitic acid is negligible in such cells, with the consequence that the oxidation of palmitic acid can be used as a reliable index of mitochondrial oxidation.

The effects of various drugs known to inhibit mitochondrial fatty acid β-oxidation in mouse or rat liver mitochondria or in isolated rat hepatocytes, were tested in this model. All these drugs also inhibited [U-^{14}C]palmitic acid oxidation by human lymphocytes (Figs 3 and 4), although IC_{50} values varied greatly (Table 1). Except for amiodarone, the IC_{50} values obtained with human lymphocytes were higher than those previously obtained with isolated mouse or rat liver mitochondria (Table 1). With VPA the IC_{50} was 0.125 mM with isolated rat liver mitochondria [2], 2.5 mM with isolated rat hepatocytes [1] and more than 10 mM with human lymphocytes (Table 1). The higher IC_{50} values in cells might reflect the need for the drug to be first taken up by cells, before reaching mitochondria. In contrast, amiodarone, which easily crosses the plasma membrane [38] and is concentrated in human lymphocytes [39], exhibited a lower IC_{50} in human lymphocytes than in mouse liver mitochondria (Table 1). Taken together, these observations suggest that human lymphocytes can be used as a reliable model to determine whether drugs inhibit mitochondrial β-oxidation in human mitochondria. IC_{50} values, however, obtained with lymphocytes may only be

valid for this particular cell, and might differ from IC_{50} values in other cells.

While all these drugs inhibited the formation of acid-soluble β-oxidation products, they elicited different effects on the formation of $[^{14}C]CO_2$ (Figs 3, 4 and 5). Indeed, three types of effects were observed. (a) Both DNP and 20 μM amiodarone increased $[^{14}C]CO_2$ production, without modifying the formation of acid-soluble β-oxidation $[^{14}C]$-products (Fig. 5). Both these substances exert an uncoupling effect [12]. The activity of the tricarboxylic acid cycle is sensitive to the NADH/NAD ratio [40, 41]. Acceleration of the mitochondrial respiration produced by uncouplers decreases this ratio, and therefore stimulates the tricarboxylic acid cycle, thus increasing $[^{14}C]CO_2$ formation [42, 43]. (b) The *R*-(−) and *S*-(+) ibuprofen enantiomers, and VPA tended to increase $[^{14}C]CO_2$ production, while inhibiting the formation of acid-soluble β-oxidation ^{14}C-products (Fig. 3). Both compounds have been shown to inhibit β-oxidation without inhibiting the tricarboxylic acid cycle [1, 14, 44]. Under these conditions, the decreased NADH formation following the inhibition of β-oxidation may also decrease the NADH/NAD ratio, and thus stimulate the tricarboxylic acid cycle (as described above), so that, despite the reduction in β-oxidation, a much higher percentage of acetyl-coenzyme A units are now directed towards $[^{14}C]CO_2$ formation [44]. (c) Tianeptine, amineptine, tetracycline and amiodarone (the latter at concentrations of 40–100 μM) decreased the formation of both $[^{14}C]CO_2$ and acid-soluble β-oxidation ^{14}C-products (Fig. 4). Tianeptine [10], amineptine [9], tetracycline [4] and amiodarone (the latter at relatively high concentrations) [12] have been found to decrease the tricarboxylic acid cycle activity in isolated mouse liver mitochondria. Inhibition of the tricarboxylic acid cycle prevents the compensatory stimulation described above, so that decreased formation of acyl-coenzyme A units due to decreased β-oxidation is associated with decreased formation of CO_2 from acetyl-coenzyme A.

In the standard system, human lymphocytes were incubated with 1 mM palmitic acid, without glucose. Under these conditions, energy from palmitate oxidation may significantly contribute to ATP formation. Indeed, there was a general trend for decreased fatty acid oxidation to be associated with decreased ATP levels (Fig. 6). With six compounds, however, namely DNP, the *R*-(−) and *S*-(+)ibuprofen enantiomers, KCN, rotenone and amiodarone, ATP depletion was somewhat higher than with other compounds which produced comparable effects on [U-^{14}C]palmitic acid oxidation (Fig. 6). Interestingly, these six compounds were either uncouplers of oxidative phosphorylation, i.e. DNP, ibuprofen [45] and amiodarone [12] and/or inhibitors of the respiratory chain, i.e. amiodarone [12], rotenone and KCN [35]. Uncoupling of oxidative phosphorylation and/or inhibition of the respiratory chain should lead, in both cases, to further impairment of ATP formation.

Amiodarone produces four effects on mitochondrial function (uncoupling, inhibition of β-oxidation, inhibition of the tricarboxylic acid cycle and inhibition of respiration) [11, 12] and led to quick depletion of ATP and loss of viability (Figs 7 and 9). Similar decreases in ATP and viability have been reported, in abstract form [46]. Addition of 5 mM glucose which can produce ATP through glycolysis despite decreased mitochondrial function, partially prevented amiodarone-induced ATP depletion and toxicity (Table 3). These beneficial effects were absent if iodoacetate, a glycolysis inhibitor was concomitantly added (Table 3). Thus, ATP depletion was probably involved, at least in part, in the cytotoxicity of amiodarone towards lymphocytes. Two observations suggest that some other mechanism(s) may have also been involved. First, glucose only partially prevented the cytotoxicity of amiodarone (Table 3). Second, the decrease in viability due to 100 μM amiodarone could not be reproduced when lymphocytes were incubated with tetracycline (4 mM), rotenone (30 μM) and DNP (160 μM), a combination which reproduces the various mitochondrial effects of amiodarone. In addition to its effects on mitochondria, amiodarone alters lipid dynamics in membranes, ion transport mechanisms, calcium-signaling systems, and inhibits Na^+,K^+-ATPase [38]. Some of these additional effect(s) may contribute, together with ATP depletion, to the lymphocytotoxic effects of amiodarone.

During chronic treatment, the steady-state serum concentration of amiodarone plus *N*-desethyl-amiodarone may reach 10 μM in some human recipients [47]. In the present study, 20 μM amiodarone produced some toxicity in lymphocytes incubated without glucose (Fig. 8). Although lymphocytes *in vivo* may be protected by the availability of glucose, a study to determine the possible *in vivo* effects of amiodarone on lymphocyte function in humans may be warranted.

In summary, human lymphocytes may be a useful and convenient model to assess the effects of drugs on [U-^{14}C]palmitic acid oxidation by human mitochondria. This technique allows a rapid determination of whether a drug inhibits fatty acid oxidation in human mitochondria. IC_{50} values determined with human lymphocytes, however, may not necessarily apply to other cells.

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