Fatty-acid-binding proteins do not protect against induced cytotoxicity in a kidney cell model

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Intracellular accumulation of fatty acids (FAs) is a well-described consequence of renal ischaemia and may lead to lethal cell injury. Fatty-acid-binding proteins (FABPs) are small cytosolic proteins with high affinity for FAs. They may protect vital cellular functions by binding to and promoting the metabolism of FAs, thereby reducing their intracellular concentration. In this study we investigated the putative cytoprotective role of FABPs in a Madin–Darby canine kidney (MDCK) cell model for renal damage. We studied the effects of transfection with cDNA encoding heart FABP, adipocyte FABP or liver FABP on cytotoxicity induced by chemical anoxia or FAs. Transfection of MDCK type II cells with these cDNA types caused a 5–20-fold increase in FABP content, but did not change the rate or extent of palmitate uptake. After 1 h of incubation with KCN, all cell types showed reduced viability and cellular ATP content and an intracellular accumulation of non-esterified FAs. High extracellular concentrations of oleate, but not palmitate, caused a markedly decreased cell viability and cellular ATP content. Oleate accumulated in non-esterified form in these cells. Simultaneous addition of glucose ameliorated the damaging effects of KCN or oleate, indicating that glycolytic ATP could substitute for uncoupled oxidative phosphorylation. No significant differences in the effects of chemical anoxia or oleate were observed between non-transfected, mock-transfected and FABP-cDNA-transfected cells. Non-esterified FA accumulation was not reduced in any of the FABP-cDNA-transfected cell lines. In conclusion, our data do not provide evidence for a cytoprotective role of FABP in this kidney cell model.

Key words: chemical anoxia, cytoprotection, fatty acid accumulation, ischaemia, Madin–Darby canine kidney cells.

INTRODUCTION

Ischaemia and hypoxia are major causes of renal failure. Intracellular accumulation of non-esterified fatty acids (FAs) is a well-described consequence of renal ischaemia [1], and ATP depletion is induced by chemical anoxia [2]. Multiple phospholipases are probably activated by Ca^{2+} as a result of ATP depletion, but the large amounts of FAs and lysophospholipids released after ischaemia indicate that phospholipase A_2 is responsible for most of the FAs released [1,3].

Activation of this enzyme and the resulting release and accumulation of FAs have been implicated in the pathogenesis of cell injury after renal ischaemia [4,5]. In proximal tubule segments from mouse, hypoxia caused an approx. 8-fold increment of FAs [6]. Similarly, FAs accumulated when cultures of mouse proximal tubule cells and Madin–Darby canine kidney (MDCK) cells were subjected to chemical anoxia [7,8]. Extracellular albumin reduced accumulation of FAs and ameliorated injury, mitochondrial function and cell viability in renal tubules after ischaemia [7]. It has also been proposed that accumulation of FAs (especially arachidonic acid) can result in feedback inhibition of phospholipase A_2, thereby protecting against hypoxic injury [6,9].

Fatty-acid-binding proteins (FABPs) are 14–16 kDa cytosolic proteins, involved in the intracellular binding and transport of long-chain FAs. They bind FAs with K_d values between 2 and 1000 nM, depending on the type of FABP and the type of FA [10,11]. FABPs may modulate the role of FAs in cellular processes, like metabolism, signal transduction and gene expression, and the function of ion channels, receptors and enzymes [12–15].

In addition to these functions FABPs may have cytoprotective effects, e.g. in the cardiomyocyte [12]. It has been hypothesized that FABPs protect vital cellular functions by binding intracellular long-chain FAs and their derivatives [12,14–16]. FABPs are suitable for this function since they are present intracellularly in concentrations considerably higher than those of long-chain FAs [14,17]. Vork et al. [18,19] suggested that heart FABP (H-FABP) can bind the FAs accumulating under pathophysiological circumstances, and that initially the accumulating FAs are released from the cardiomyocyte in this form. Other mechanisms by which H-FABP could protect the heart include the scavenging of free radicals [20] and the inhibition of a β-adrenergic response, as observed in cultured neonatal rat heart cells [21]. The presence of FABP would enable a more rapid exchange of FAs among subcellular and intercellular sites to counteract local FA accumulation [22]. Another example of putative cytoprotection by FABP is the reversion of FA-mediated inhibition of brain synaptosomal Na^+-dependent amino acid-uptake systems by liver FABP (L-FABP) [23]. Overexpression of epidermal FABP in chemoresistant pancreatic cancer cell lines was suggested to be part of a mechanism of sequestration or removal of cytotoxic drugs [24]. Taken together, the present data do not yet allow a definitive conclusion to be drawn about the potentially protective effect of FABP.

In the present study we investigate the putative cytoprotective role of FABP against induced cytotoxicity in a MDCK cell model for renal damage. In the human kidney H-FABP and L-FABP are present in different concentrations and with differing localizations [25]. H-FABP is present in distal tubules, whereas L-FABP is identified only in proximal tubules [25]. MDCK type

Abbreviations used: FABP, fatty-acid-binding protein; A-FABP, adipocyte FABP; H-FABP, heart FABP; L-FABP, liver FABP; DOG, deoxyglucose; FA, fatty acid; MDCK, Madin–Darby canine kidney; D-PBS, Dulbecco's modified PBS.

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II epithelial cells resemble nephron distal-tubule segments [26] and were used previously to study susceptibility to chemical anoxia [7,8]. Therefore we studied the effects of transfection of MDCK type II cells with cDNA encoding three different FABP types, H-FABP, adipocyte FABP (A-FABP) and L-FABP, on KCN-induced anoxia, FA-induced cytotoxicity and FA accumulation.

EXPERIMENTAL

Materials

Puromycin dihydrochloride was from Sigma (St Louis, MO, U.S.A.); Dulbecco's modified Eagle's medium was from Gibco (Hoofddorp, The Netherlands); fetal bovine serum was from Integro (Zaandam, The Netherlands); [1-^{14}C]oleic acid (56 mCi/mmol) and [1-^{14}C]palmitic acid (57 mCi/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.); Optifluor was from Packard (Groningen, The Netherlands); trypsin was from Difco Laboratories (Detroit, MI, U.S.A.); and TLC aluminium sheets with silica gel 60 F_{254} were from Merck (Darmstadt, Germany). MDCK type II cells were a kind gift from Dr P. Deen (Department of Cell Physiology, University of Nijmegen, Nijmegen, The Netherlands). All other reagents were of analytical grade.

Culture media, buffers and cell culture

Dulbecco's modified PBS (D-PBS) contained 145 mM NaCl/5.4 mM KCl/5 mM Na_2HPO_4/25 mM glucose/25 mM sucrose (pH 7.4). Growth medium was composed of Dulbecco's modified Eagle's medium with 10 % fetal bovine serum.

MDCK type II cells were cultured in growth medium. At confluency the cells were washed with D-PBS, treated with 2 mM EDTA in D-PBS for 1 min, and trypsinized (0.05 % trypsin in D-PBS for 7–10 min at 37 °C), collected by centrifugation and seeded again in fresh growth medium. Medium was changed every 3–4 days.

Preparation of FABP expression constructs

The constructs were based on the pSG5 expression plasmid [27]. Transcription of the cDNAs was under the control of a simian virus 40 early promoter, and the construct contained a rabbit β-globin intron II and a simian virus 40 poly(A)$^+$ signal [27]. A modified pSG5 plasmid, with an extended multiple cloning site and a 1.7 kb *pac* cassette (puromycin acetyltransferase) ligated into the *Sal*I site of the multiple cloning site was used as the expression construct.

Rat H-FABP and A-FABP cDNAs were cloned into the pSG5 expression plasmid as described previously [28]. Human L-FABP cDNA in the prokaryotic expression plasmid pET3b was cloned as described by Maatman et al. [29]. The *Eco*RI site in the L-FABP cDNA was removed by the unique site elimination kit (Amersham Pharmacia Biotech) using the following primer: 5′-GTGATCCAAAACGAGTTCACGGTGGGGGAG-3′. Codon GAA encoding Glu-62 was replaced by GAG, which does not induce a change at the amino acid level. Human L-FABP was subsequently amplified using primers with recognition sites for *Eco*RI, to facilitate cloning into the *Eco*RI site of the pSG5-*pac* expression plasmid's multiple cloning site: forward primer, 5′-TAGGGCGAATTCGGCTTACATATGAGTTTC-3′; reverse primer, 5′-CCCATCGAATTCGGCTCCGGATCCTGTTTA-3′. The orientation of the FABP insert was verified by *Pst*I/*Kpn*I digestion and automatic DNA sequencing.

Introduction of plasmids into MDCK type II cells by $Ca_3(PO_4)_2$ transfection

MDCK type II cells were seeded at a density of 1×10^6 cells/100 mm dish, and cultured for 18 h to 40–50 % confluency. Medium was refreshed and after 2 h transfection was started. Linearized DNA (25–30 μg) was mixed with 125 mM $CaCl_2$ in HBS buffer (140 mM NaCl/5 mM KCl/0.75 mM Na_2HPO_4/6 mM dextrose/25 mM Hepes, pH 7.05). The DNA suspension was added dropwise to the cells and incubated for 6 h at 37 °C under 5 % CO_2. Cells were shocked by addition of 20 % glycerol in TBS buffer (137 mM NaCl/2.7 mM KCl/0.6 mM Na_2HPO_4/0.1 M Tris/0.75 mM $CaCl_2$/0.75 mM $MgCl_2$, pH 7.5) and incubated for 2 min at 22 °C. Subsequently cells were washed with TBS buffer and fresh growth medium was added to the cells. After 24 h incubation at 37 °C with 5 % CO_2, cells were trypsinized and seeded in six-well culture plates in growth medium containing 5 μg/ml puromycin. Medium was refreshed every 3 days. Then, 7–10 days after transfection, separate colonies were picked and grown to check for clonal variation.

SDS/PAGE and immunoblotting

Cytoplasmic cell fractions were isolated by homogenization in SET buffer (250 mM sucrose/2 mM EDTA/10 mM Tris, pH 7.4) and subsequent centrifugation for 15 min at 14000 ***g*** at 4 °C. Cytoplasmic fractions were analysed for FABP expression by separation of 10 μg of protein by SDS/PAGE (12.5 % gel) and Western blotting. Rat H- or A-FABP and human L-FABP were detected with polyclonal rabbit antisera and the ECL detection system (Amersham Pharmacia Biotech). FABP contents were determined by ELISA. Polyclonal antisera (1:500 dilutions) were used as first antibodies. Peroxidase-conjugated goat anti-rabbit IgG (γ-chain specific, 1:5000 dilution) was used as the second antibody. Absorbance was measured at 490 nm. Purified recombinant rat A-FABP and H-FABP, and human L-FABP with binding characteristics comparable with tissue-derived or recombinant proteins were used as standards [28,29].

Assay of palmitic acid uptake

Palmitic acid uptake was determined as described previously for L6 myoblasts [28]. Incubation with 75 μM [1$-^{14}$C]palmitic acid bound to 15 μM albumin was performed for 1–30 min. At each time point three plates were assayed. Values were corrected for a blank that was determined by aspirating the FA-containing solution immediately after addition. The data were normalized for protein content, determined on three identical plates. Cells were washed three times with PBS, lysed with 0.5 M NaOH, neutralized with 6 M HCl and used for protein determination according to Lowry et al. [30].

Methods to induce cytotoxicity

Chemical anoxia

Chemical anoxia was induced as described previously [7]. Triplicate cell monolayers were incubated for 1 h with 10 mM KCN or 10 mM KCN/5 mM deoxyglucose (DOG) in Krebs–Henseleit buffer (115 mM NaCl/3.6 mM KCl/1.3 mM KH_2PO_4/25 mM $NaHCO_3$, pH 7.4), in the presence or not of 10 mM glucose without metabolic inhibitors at 37 °C under 5 % CO_2. Incubation in the presence of 10 mM glucose only was used as a control.

Exogenous FAs

Oleic acid and palmitic acid were dissolved in 96 % ethanol. A stock concentration of 50 mM was divided into aliquots and

frozen at −20 °C. On the day of the experiment, aliquots were diluted to 1:10 or 1:4, respectively, in 96% ethanol, and further diluted in Krebs–Henseleit buffer to 50 and 125 μM for both oleic and palmitic acids. Cells were grown to confluence in 24-well culture plates in growth medium and were subsequently incubated for 1 h at 37 °C under 5% CO_2 with either 50 or 125 μM oleic or palmitic acid in Krebs–Henseleit buffer, in the presence or not of 10 mM glucose. The ethanol concentration during the experiment was 0.1%. Incubation in the presence of 10 mM glucose only was used as a control.

Evaluation of cell injury

Cell viability

Cells from duplicate wells (grown in 24-well culture plates) were harvested at the end of each period of induced injury. Cells were centrifuged at 185 ***g*** for 5 min and resuspended in 100 μl of growth medium. Trypan Blue (0.25%) in PBS was added, and the number of viable cells (excluding Trypan Blue) was counted and expressed as the percentage of total cells.

Cell ATP content

Cell ATP was extracted with 4% perchloric acid at 0 °C. ATP levels were measured using a luciferase assay with purified luciferin/luciferase (Promega, Madison, WI, U.S.A.). Cell ATP levels were expressed in nmol/mg of cell protein.

Measurement of FA accumulation

Cells were grown to confluence in 60 mm dishes in culture medium for 36 h in the presence of 50 μM [1-^{14}C]palmitic acid bound to 12.5 μM albumin and washed with PBS. After a subsequent incubation for 1 h with 10 mM KCN in Krebs–Henseleit buffer at 37 °C under 5% CO_2, the cells were washed three times with 0.15 M NaCl, centrifuged for 5 min at 500 ***g***, and resuspended in the same medium (2–5 mg of protein/ml).

Alternatively, confluent cells were incubated for only 1 h with different concentrations of [1-^{14}C]oleic acid in Krebs–Henseleit buffer at 37 °C under 5% CO_2. Cell extracts were prepared and analysed for lipids by TLC as described by Mazière et al. [31]. Neutral lipids were separated in hexane/diethylether/acetic acid (35:15:1, by vol.). The incorporation and accumulation of [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid were determined by liquid scintillation counting of separated spots.

RESULTS

Transfection of MDCK type II cells with cDNAs encoding different FABP types

Endogenous H-FABP and L-FABP contents of MDCK type II cells were low (< 0.05% of cytoplasmic protein) or not detectable, respectively, as determined by semi-quantitative Western blotting with specific antisera [28,32]. After stable $Ca_3(PO_4)_2$ transfection of MDCK type II cells with FABP cDNAs, separate colonies were picked and grown to check for clonal variation. Clonal variation was monitored by SDS/PAGE and subsequent immunoblotting of cytosolic fractions isolated from transfected cells (Figure 1). Among the seven isolated rat H-FABP transfectants, one clone (Figure 1A, lane 4) showed an 8-times higher expression of H-FABP than mock-transfected cells. After transfection with rat A-FABP cDNA, two of the seven isolated clones showed expression of A-FABP, but in only one clone was the expression 4 times higher than the mock-transfectant (Figure 1B, lane 3). Of 15 isolated clones transfected with human L-FABP cDNA, six showed increased L-FABP expression (Figure 1C).

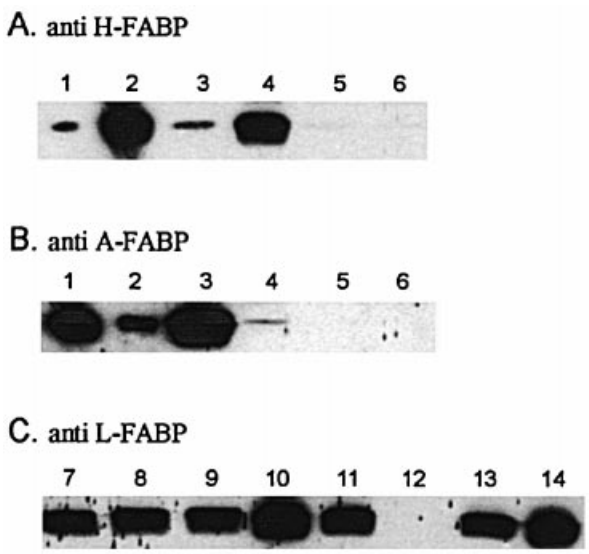

Figure 1 FABP expression in non-transfected and transfected MDCK II clones

Cytosolic protein (10 μg) was separated by SDS/PAGE (12.5% gel). After Western blotting on nitrocellulose, detection was performed with polyclonal antisera (1:1000 dilution) directed against rat H-FABP (**A**), A-FABP (**B**) or L-FABP (**C**). Recombinant H-, A- or L-FABP (100 ng) were used as standards. (**A**, **B**) Lane 1, A-FABP; lane 2, H-FABP; lane 3, A-FABP cDNA-transfected clone 5; lane 4, H-FABP cDNA-transfected clone 4; lane 5, mock-transfected clone 6; lane 6, non-transfected cells. (**C**) Lanes 7–13, L-FABP cDNA-transfected clones 1, 2, 4, 5, 6, 7, 9 respectively; lane 14, 100 ng of L-FABP.

Table 1 FABP content in mock-transfected and FABP cDNA-tranfected MDCK type II clones

FABP contents were determined by ELISA using purified recombinant H-, A- or L-FABP as standards. Polyclonal antisera (1:500 dilutions) were used as first antibodies. Peroxidase-conjugated goat anti-rabbit IgG (γ-chain specific, 1:5000 dilution) was used as the second antibody. Absorbance was measured at 490 nm. Values are means ± S.D. from at least three independent experiments. –, Not determined.

	FABP content (μg/mg of protein)		
Clone	H-FABP	A-FABP	L-FABP
Mock 1	–	–	0.5 ± 0.3
Mock 6	1.3 ± 0.4	1.5 ± 0.8	0.6 ± 0.3
Mock 7	1.4 ± 0.4	1.8 ± 0.7	–
Mock 8	1.4 ± 0.3	1.8 ± 0.7	–
Mock 9	–	–	0.5 ± 0.1
H-FABP 4	11.4 ± 1.9		
H-FABP 6	1.7 ± 0.2		
H-FABP 7	2.1 ± 0.1		
A-FABP 5		6.8 ± 0.9	
A-FABP 7		1.8 ± 0.8	
L-FABP 1			4.6 ± 1.5
L-FABP 2			4.9 ± 1.4
L-FABP 4			5.2 ± 1.3
L-FABP 5			15.6 ± 4.0
L-FABP 6			5.7 ± 1.1
L-FABP 9			4.8 ± 1.0

Table 2 Uptake of [1-^{14}C]palmitic acid by non-transfected and transfected MDCK type II cells

Confluent cultures on growth medium were used, and cells were equilibrated for 1 h at 37 °C under 5% CO_2 on Dulbecco's modified Eagle's medium without serum. The assay was started by the addition of 75 μM [1-^{14}C]palmitic acid bound to 15 μM albumin. Values are means ± S.D. from three independent experiments performed in triplicate. No significant differences were detected between the different clone types.

		[1-^{14}C]Palmitic acid uptake (nmol of palmitic acid/mg of protein)			
Clone type	Time point (min) ...	1	5	15	30
Non-transfected		2.7 ± 0.1	4.8 ± 0.1	6.6 ± 0.9	15.4 ± 2.5
Mock-transfected		3.2 ± 0.6	5.1 ± 0.6	7.3 ± 2.0	15.0 ± 6.1
H-FABP cDNA		2.3 ± 0.9	4.4 ± 0.3	8.0 ± 1.8	14.2 ± 5.0
A-FABP cDNA		2.3 ± 0.2	4.5 ± 0.2	8.3 ± 1.7	14.3 ± 6.3
L-FABP cDNA		3.3 ± 0.3	5.7 ± 0.4	9.9 ± 0.8	14.3 ± 5.0

Table 3 Comparison of the effects of chemical anoxia (KCN), the combination of KCN and DOG or glucose, and exogenous oleate or palmitate on cell viability in MDCK type II cells, transfected or not with cDNAs encoding different FABP types

Cells were incubated for 1 h with 10 mM glucose (controls) or with the indicated agent. Glucose-treated control cells showed between 83 and 98% viable cells as determined by Trypan Blue staining. Viability is given for all cell types as a percentage of the appropriate control (means ± S.D. from three independent experiments).

	Viability (% of control)							
				Oleate		Palmitate		
Cell type	10 mM KCN	10 mM. KCN/ 5 mM DOG	10 mM KCN/ 10 mM glucose	50 μM	125 μM	50 μM	125 μM	125 μM Oleate/ 10 mM glucose
MDCK II	62 ± 40	73 ± 15	88 ± 15	32 ± 16*	8 ± 7*	85 ± 20	89 ± 18	62 ± 24†
Mock	58 ± 17‡	54 ± 11‡	87 ± 8	31 ± 20*	0 ± 0*	94 ± 7	80 ± 21	50 ± 17†
H-FABP	61 ± 11‡	62 ± 12‡	98 ± 6	22 ± 4*	0 ± 0*	95 ± 9	82 ± 11	48 ± 15†
A-FABP	50 ± 23	54 ± 33	73 ± 28	44 ± 25*	0 ± 0*	91 ± 12	62 ± 36	75 ± 14†
L-FABP	37 ± 18§	36 ± 30	76 ± 17	34 ± 19*	4 ± 6*	79 ± 24	60 ± 17	47 ± 20†

* $P < 0.01$ as compared with control.
† $P < 0.05$ as compared with oleate alone.
‡ $P < 0.05$ as compared with KCN/glucose.
§ $P < 0.1$ as compared with KCN/glucose.

The FABP contents in transfected clones were also determined by ELISA (Table 1). The contents of H-FABP, A-FABP and L-FABP in mock-transfected cells were in the same range as in non-transfected MDCK type II cells. In rat H-FABP cDNA-transfected clone 4, H-FABP expression was significantly increased compared with mock-transfected cells. The L-FABP content in all L-FABP cDNA-transfected clones studied was significantly higher than in mock-transfected cells. A-FABP cDNA-transfected clone 5 contained a significantly higher amount of A-FABP than the mock-transfectants.

All transfected clones were morphologically similar to non-transfected MDCK type II cells, and showed no differences in growth rate compared with mock-transfected or non-transfected cells. Single representative clones with high expression of H-FABP (clone 4), A-FABP (clone 5) or L-FABP (clone 5) and one mock clone (clone 6) were used in the following studies.

Palmitic acid uptake

We did not observe significant differences in palmitic acid uptake of the different transfected MDCK II cultures after 1–30 min (Table 2). H-FABP, A-FABP or L-FABP transfection and overexpression did not have an effect on the palmitic acid uptake (after 1 min) or uptake together with metabolism (longer periods).

Effects of chemical anoxia on cell viability and ATP content

Chemical anoxia, induced by 1 h incubation with 10 mM KCN, resulted in a decreased number of viable cells for all cell types (Table 3). The same effect was observed when 10 mM KCN was added in combination with 5 mM DOG. The decrease in cell viability induced by KCN could be reduced by co-incubation with 10 mM glucose, although this effect was not significant for the non-transfected and the A-FABP-cDNA-transfected cells. No significant differences in the effect of chemical anoxia were observed between non-transfected, mock-transfected and FABP-cDNA-transfected cells.

Cells treated for 1 h with 10 mM glucose (control) contained 6.4 ± 2.6 nmol of ATP/mg of cytosolic protein (mean ± S.D., $n = 24$). This value did not differ between mock-transfected, FABP cDNA-transfected and non-transfected cells. Incubation of the clones with 10 mM KCN generally resulted in a reduction of ATP content to 43–60% of the control (Table 4). Addition of 10 mM KCN together with 10 mM glucose had no effect compared with the control. Treatment with a combination of 10 mM KCN and 5 mM DOG led to a marked decrease of ATP content in all cell types (7–25% of the control).

To establish the probable cause of the cellular injury, we studied the accumulation of labelled non-esterified FAs in cells cultured for 36 h with [1-^{14}C]palmitate and subsequently incubated for 1 h with 10 mM KCN in Krebs–Henseleit buffer (Table

Table 4 Comparison of the effects of chemical anoxia (KCN), the combination of KCN and DOG or glucose, and exogenous oleate or palmitate on ATP content in MDCK II cells, either transfected or not with cDNA encoding different FABP types

Cells were incubated for 1 h with 10 mM glucose (controls) or with the indicated agents. The ATP content of glucose-incubated cells was 6.4 ± 2.6 nmol/mg of cytosolic protein and did not differ for the various transfected cells. The ATP content of treated cells is given as a percentage of the appropriate control (means ± S.D. from three independent experiments).

	ATP content (% of control)							
				Oleate		Palmitate		
Cell type	10 mM KCN	10 mM KCN/ 5 mM DOG	10 mM KCN/ 10 mM glucose	50 μM	125 μM	50 μM	125 μM	125 μM Oleate/ 10 mM glucose
MDCK II	60 ± 28‡	9 ± 1‡	116 ± 5	48 ± 11*	4 ± 3*	85 ± 13	91 ± 16	94 ± 22†
Mock	43 ± 29‡	25 ± 7‡	125 ± 13	13 ± 8*	2 ± 1*	93 ± 14	70 ± 11	66 ± 16†
H-FABP	52 ± 35	11 ± 6‡	90 ± 25	12 ± 6*	2 ± 1*	107 ± 11	92 ± 16	79 ± 5†
A-FABP	49 ± 20‡	7 ± 0‡	102 ± 28	16 ± 14*	0 ± 0*	73 ± 31	71 ± 9	70 ± 39†
L-FABP	49 ± 24‡	8 ± 2‡	109 ± 24	23 ± 19*	0 ± 0*	106 ± 33	73 ± 22	86 ± 48†

‡ $P < 0.05$ as compared with KCN/glucose.
* $P < 0.01$ as compared with control.
† $P < 0.05$ as compared with oleate alone.

Table 5 Accumulation of non-esterified FAs in non-transfected and transfected MDCK II clones after induction of chemical anoxia

Cells were cultured for 36 h on growth medium with 50 μM [1-^{14}C]palmitic acid bound to 12.5 μM albumin, washed and subsequently incubated for 1 h in Krebs–Henseleit buffer with or without 10 mM KCN. Cell homogenates were analysed by TLC. FA spots were isolated and counted by liquid scintillation. Values are means ± S.D. from three independent experiments.

	Intracellular palmitic acid (pmol/mg of cell protein)	
Cell type	10 mM KCN	Control
Non-transfected	169 ± 2	138 ± 11
Mock-transfected	246 ± 17	168 ± 14
H-FABP cDNA	157 ± 8	130 ± 1
A-FABP cDNA	231 ± 7	147 ± 11
L-FABP cDNA	211 ± 9	138 ± 3

Table 6 Oleic acid uptake and incorporation into neutral lipids by MDCK cells

Cells were incubated for 1 h with 10, 50 or 125 μM [1-^{14}C]oleic acid in Krebs–Henseleit buffer at 37 °C under 5% CO_2. Cell homogenates were analysed by TLC. Cholesterol (C) + diacylglycerol (DG), triacylglycerol (TG) and FA spots were isolated and counted by liquid scintillation. Values are means ± range from two independent experiments. Similar values were obtained for FABP-cDNA-transfected and mock-transfected cells.

	Oleic acid uptake and incorporation (pmol/mg of cell protein)		
Oleic acid concentration (μM)	C + DG	TG	FA
10	9 ± 1	50 ± 8	37 ± 9
50	25 ± 1	729 ± 65	256 ± 83
125	13 ± 1	385 ± 120	1436 ± 276

5). The palmitic acid release was taken as representative for non-esterified FAs. No marked differences were observed in FA incorporation among the five different cell types after culturing (results not shown). In all KCN-treated cells the content of labelled non-esterified palmitic acid became markedly higher than in non-treated cells (Table 5).

Effect of oleic or palmitic acid on cell viability and ATP content

Exposure for 2 h of the cells to oleic acid (125 μM) completely eliminated cell viability, whereas after a 1 h exposure a reduction to 8% of the control (10 mM glucose) was achieved (Table 3). No effect was observed after 1 h incubation with 10 μM oleic acid (results not shown). Incubation with 50 μM oleic acid reduced cell viability of the non-transfected and transfected cells to 22–44%. The effect of 125 μM oleic acid could be suppressed by co-incubation with 10 mM glucose. In contrast with oleic acid, palmitic acid did not significantly affect cell viability of all cell types used, independent of its concentration. No BSA was included during the incubations, so micelle formation most assuredly took place. However, it did not result in cell-membrane disruption, as observed by light microscopy.

Treatment of cells with 50 μM oleic acid for 1 h resulted in a decreased ATP content, varying between 12 and 48% of the control (Table 4). After treatment with 125 μM oleic acid no ATP could be detected. The ATP content could be recovered to 66–94% of control levels by co-incubation with 10 mM glucose. In contrast with oleic acid, palmitic acid did not affect or only to a slight extent affected ATP content in all cell types used (Table 4).

Comparison of non-transfected cells with FABP cDNA-transfected cells showed no significant differences in cell viability and ATP content after incubation with 50 or 125 μM oleic acid (Tables 3 and 4). These results indicate that transfection of MDCK type II cells with FABP-encoding cDNA has no effect on oleic acid-induced cytotoxicity.

To establish the extent of intracellular accumulation of FAs, incorporation of [1-^{14}C]oleic acid was measured after 1 h of incubation with concentrations between 0 and 125 μM in Krebs–Henseleit buffer. Intracellular non-esterified oleic acid was significantly higher in MDCK cells treated with 125 μM oleate than in those treated with 10 or 50 μM oleate (Table 6). Incorporation of oleate into diacylglycerols/cholesterol and triacylglycerols was, however, lower at 125 μM than at 50 μM, presumably by ATP deficiency. The radioactivity of the cholesterol + diacylglycerol spot was mainly in diacylglycerol, which migrated together with cholesterol on TLC. No differences were observed between mock-transfected, FABP-cDNA-transfected and non-transfected cells (results not shown). These observations indicate uptake and accumulation of non-esterified oleate in all cell types. The intracellular non-esterified oleate concentration was related to the extracellular concentration. The presence of FABP did not

cause a lower intracellular concentration of non-esterified oleic acid.

DISCUSSION

Although previous studies have suggested a protective role of FABP against high intracellular FA concentrations [12,14–16,18,19], free radicals [20] or cytotoxic drugs [24], evidence for such a role is lacking. In this study we investigated the putative cytoprotective role of FABPs in a kidney cell model.

The kidney contains two types of FABP: the heart and liver types. L-FABP is twice as abundant in the human kidney than H-FABP, but is predominantly localized in cells of the proximal tubules. H-FABP is mainly present in the distal tubule cells [25]. In rat kidney the H-FABP content is 10 times higher than the L-FABP content [25,33]. Like in humans, H-FABP is only present in the distal tubules, but L-FABP is present in proximal and distal tubules [33]. In agreement with these findings, we detected a higher content of endogenous H-FABP than of L-FABP in MDCK type II cells, which are epithelial cells originating from the distal tubule. Transfection of MDCK type II cells with H-FABP, A-FABP or L-FABP cDNA caused a 5–20-fold increase in cytoplasmic FABP content, as shown by Western-blot analysis and ELISA. The concentration of H-FABP in transfected MDCK cells is about equal to that in rat and human kidney (10 and 5 μg/mg, respectively [33]). The L-FABP concentration is higher after transfection than in human and rat kidney (9 and 0.9 μg/mg, respectively [33]).

The increase of the H-FABP, A-FABP or L-FABP content in MDCK type II cells did not cause a difference in the rate or extent of palmitate uptake after 1–30 min. Comparable results were obtained with L6 myoblasts overexpressing H-FABP or A-FABP [28]. Expression of L-FABP in mouse L-cell fibroblasts increased the initial rate and extent of *cis*-parinaric acid uptake by 50 %, whereas expression of intestinal FABP did not affect FA uptake [34,35]. However, in L-cells expressing larger amounts of intestinal FABP, FA uptake decreased [36]. In a differentiated enterocyte model high levels of intestinal FABP also inhibited FA incorporation [37]. A direct correlation between L-FABP content and FA uptake was observed in HepG2 cells either transfected with antisense L-FABP mRNA or treated with a peroxisome proliferator [38]. In a human breast cancer cell line transfected with H-FABP cDNA a modest increase in palmitic and oleic acid uptake was observed, but there was no increase of the incorporation into phospholipids or triacylglycerols [39]. Taken together, these observations suggest that the effect of FABP on FA uptake and incorporation may differ according to the cell type used and the FABP type expressed.

We investigated the effect of FABP cDNA transfection of MDCK cells on KCN- and FA-induced cytotoxicity. KCN inhibits oxidative phosphorylation in mitochondria and causes chemical anoxia. We observed marked and similar decreases in cellular ATP content after KCN addition to FABP cDNA-transfected and non-transfected cells (Table 4). The decrease in cell viability was modest and was significant only for mock- and H-FABP cDNA-transfected cells (Table 3). These findings are in agreement with a critical ATP level (5–15 % of the control) above which cellular function and integrity are maintained [2,7]. Addition of DOG together with KCN had an even stronger effect on the ATP content, since DOG inhibits the glycolysis as well. MDCK cells do demonstrate the ability to generate ATP via a glycolytic metabolism, since the addition of glucose ameliorated the severity of ATP depletion. Resistance of continuous cell lines to ischaemic injury by glycolytic capacity has been reported previously [2,7].

We demonstrated that ATP depletion by KCN is accompanied by accumulation of non-esterified FAs in MDCK cells (Table 5). After 1 h of incubation with KCN, the amount of intracellular palmitate was increased by approx. 1.5-fold compared with the controls. Sheridan et al. [7] reported a comparable accumulation of palmitate, and also of palmitoleate, stearate and oleate in MDCK cells after KCN addition.

Cytotoxicity was also induced by extracellular addition of high concentrations of oleate (Tables 3 and 4). Oleate in combination with glucose resulted only in a modest decrease in cell viability and no reduction of ATP levels, in agreement with previous experiments [8]. Our data show that the intracellular concentration of non-esterified oleic acid was related to the extracellular concentration (Table 6). Intracellular accumulation of FAs under pathophysiological conditions may decrease the intracellular ATP content by uncoupling of oxidative phosphorylation by different mechanisms. At high concentrations, FAs probably irreversibly disrupt the membrane in a detergent-like mode [40,41]. We found, however, that addition of glucose together with oleate resulted in significant increases in cell viability and ATP content (Tables 3 and 4), indicating that glycolysis occurs and that cell injury by oleate is not only due to a detergent effect. Other mechanisms of FA-mediated uncoupling are their protonophoric action on the inner mitochondrial membrane and their interference with the proton-pumping machinery of the respiratory chain [41]. At low concentrations, FAs may also act indirectly via uncoupling proteins which dissipate the proton gradient across the mitochondrial membrane [42]. FAs activate uncoupling protein 1 (UCP1) by acting as second messengers [43]. Oleic acid also activates the *UCP*3 gene in C_2C_{12} myoblasts in a time- and dose-dependent manner [44].

Differences have been found in the effect of oleic and palmitic acid on cellular behaviour. Oleate was reported to cause an intracellular release of Ca^{2+} in platelets, a phenomenon that plays a role in the pathogenesis of ischaemic cell injury [45]. However, stearate and palmitate had no effect on the cytosolic Ca^{2+} concentration. Furthermore, it has been shown that unsaturated FAs, in contrast with saturated FAs, could increase the membrane fluidity in cytotoxic T-lymphocytes [46]. A role for oleate, but not palmitate, in respiratory control and enzyme activity was suggested, since oleate influences the membrane potential in cytochrome *c* oxidase-containing phospholipid vesicles [47]. In agreement with the above-mentioned findings, we observed that exposure of MDCK cells to oleate resulted in a loss of cell viability and ATP content in a dose-dependent manner, whereas palmitate had no effect (Tables 3 and 4).

Although FABPs were proposed to have a cytoprotective function by reducing high intracellular FA levels, the FABP cDNA-transfected MDCK cells showed similar cytotoxicity, ATP depletion, and FA uptake and accumulation to non-transfected and mock-transfected cells. These results indicate that FABPs do not substantially reduce intracellular levels of non-esterified FAs in these cells, in spite of their possible role in transfer and metabolism. In contrast, extracellular albumin (3 %) ameliorated cell injury in mouse proximal tubule cells at addition of cyanide and DOG and reduced the amount of intracellular non-esterified FAs found after ATP depletion [7]. Extraction of FAs from cells by the large amount of albumin can explain the counteracting effect.

In conclusion, our data provide no evidence for a cytoprotective role of FABP in the kidney cell model used. None of the FABP cDNA types used for transfection of MDCK type II cells were able to decrease the deleterious effects of the intracellular accumulation of FAs, caused by extracellular addition of KCN and DOG or oleic acid.

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