Branched Chain Fatty Acids Induce Nitric Oxide-dependent Apoptosis in Vascular Smooth Muscle Cells*

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Clinical observations in patients with peroxisomal disorders and studies employing corresponding mouse models have shown that supraphysiological concentrations of dietary branched chain fatty acids (BCFAs) are associated with a high level of toxicity, which is poorly understood at present. Here we show that phytanic and pristanic acid, two BCFAs that are metabolized in peroxisomes, promote apoptosis in cultured vascular smooth muscle cells of human, rat, and porcine origin. Under the conditions used, the apoptosis-promoting effect of BCFAs was neither shared by saturated or unsaturated straight chain fatty acids nor by artificial peroxisome proliferators, which, like phytanic and pristanic acid, have been shown to activate the peroxisome proliferator-activated receptor α (PPARα). We could demonstrate, however, that BCA-induced tumor necrosis factor α (TNFα) activation and secretion, which is an obligatory step required for induction of apoptosis by BCFAs. Furthermore, incubation of VSMCs with BCA-induced nitric-oxide synthase (iNOS) mRNA and protein concentrations markedly within 2 h of treatment. Correspondingly, apoptosis was significantly reduced when the cells were co-treated with the competitive NOS inhibitors monomethyl-L-arginine monoacetate and aminoguanidine. Moreover, co-incubation with TGFβ1, previously shown to destabilize iNOS mRNA, also abolished apoptosis. These results establish a new signaling cascade in which natural BCA induced NO-dependent apoptosis, which is apparently triggered by autocrine secretion of TNFα in cultured VSMCs.

Phytanic acid, a degradation product of the chlorophyll side chain, is a prominent natural branched chain fatty acid (BCFA) whose most important dietary sources in humans are

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The abbreviations used are: BCA, branched chain fatty acids; iNOS, inducible nitric-oxide synthase; MFP-2, multifunctional protein-2; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; RXX, retinoid X receptor; TGF, transforming growth factor; TNF, tumor necrosis factor; VSMC, vascular smooth muscle cell; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; CAT, chloramphenicol acetyltransferase.

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which led to excessive storage of phytanic acid along with pristanic acid, were lethal for the SCP2/SCPx-null mice, whereas these diets did not affect genetically normal animals (14). These data led to the assumption that accumulation of pristanic acid, either alone or in combination with phytanic acid, may have been responsible for the observed toxicity of BCFA-containing diets in the MFP-2-deficient patients and the SCP2/SCPx-null mice. In the present study, we therefore investigated potentially toxic effects of phytanic and pristanic acid in vitro in VSMCs, which express PPARα and RXR. We could demonstrate that treatment of VSMCs with relatively moderate concentrations of phytanic and/or pristanic acid can induce apoptosis, which, to our surprise, was not related to RXR/PPAR superstimulation but to activation of a so far unknown signaling pathway leading to activation of autocrine secretion of TNFα along with a very significant induction of iNOS gene expression by phytanic and pristanic acid.

MATERIALS AND METHODS

Reagents and Chemicals—If not stated otherwise, chemicals and tissue culture media were obtained from Sigma. Aminohydroxyquinidine was purchased from Calbiochem (Darmstadt, Germany), Wy 4.643 was from Biomol (Hamburg, Germany), fetal calf serum from ICN Biomedicals (Eschwege, Germany), and pristanic acid was obtained from H. J. Tenbrink (University of Amsterdam, NL).

Cell Culture—Rat VSMCs, a gift from Dr. M. Tepel (Heme, Germany), were isolated from thoracic aortas of male Wistar rats by the method of Franks et al. (15). Human and porcine VSMCs were a gift from Dr. G. Plenz (Münster, Germany) and prepared as described in Ref. 16. VSMCs were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum supplemented with antibiotic/antimycotic supplement (Sigma) and passed with 1× trypsin/EDTA solution (human and porcine VSMCs) or 10× trypsin/EDTA solution (Sigma) in the case of rat VSMCs. Cells at passages 4–15 were used for the experiments. Human umbilical vein endothelial cells were a gift from Dr. A. Skaletz-Rorowski (Münster, Germany) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, epidermal growth factor (5 µg/liter), fibroblast growth factor (0.5 µg/liter), hydrocortisone (500 µg/liter), and endothelial growth supplement (Promocell, Heidelberg, Germany). Human monocyte-derived macrophages were a gift from P. Cullen (Münster, Germany) and cultured with RPMI 1640 containing 10% of human serum. For all incubation experiments, the indicated concentrations of the ligand were dissolved in Me2SO at a concentration of 5 × 10−6 M, incubated for different time periods with 100 µM phytanic or pristanic acid or Me2SO as control. Representative flow cytometry histograms of six independent experiments are shown. A, cells were stained with YO-PRO as described under “Materials and Methods.” B, cells were analyzed after treatment with FITC-conjugated annexin V. C, VSMCs were incubated in the presence of the indicated concentrations of pristanic acid or Me2SO. Afterward caspase-3 activity in cell extracts was determined by a fluorometric assay (see “Materials and Methods”). The caspase-3-inhibiting peptide DEVD-CHO (nibb) was added as negative control prior to incubation with the fluorescence substrate. Data represent means ± S.D. of three independent experiments each performed in triplicate.

Flow Cytometry Detection of Apoptosis—Subconfluent VSMCs were stimulated with agonists for various time intervals, washed twice with ice-cold phosphate-buffered saline and were collected by trypsinization followed by centrifugation. For the determination of annexin V binding, 5 × 105 cells were resuspended in 1 ml of a solution containing 140 mmol/liter NaCl, 10 mmol/liter Hepes pH 7.2, and 2.5 mmol/liter CaCl2 and annexin V-FITC (Bender Med-Systems Diagnostics, Vienna, Austria) according to the supplier’s instructions. The cell suspension was incubated for 30 min at room temperature and then subjected to flow cytometry. For the determination of the cell membrane permeability, 5 × 105 cells were resuspended in 1 ml of phosphate-buffered saline and incubated for 30 min with YO-PRO (Molecular Probes, Leiden, The Netherlands) at a final concentration of 0.1 mmol/liter as described in Ref. 18. All flow cytometric measurements were performed on a Coulter Epics Elite flow cytometer equipped with a 15 milliwatt argon ion laser (Coulter Corporation, Hialeah, FL) (excitation wavelength, 488 nm; fluorescence emission, 525 nm; data rate, ~300 cells/s).

Caspase-3 Activity Measurements—Cells were harvested in a buffer consisting of 25 mmol/liter Hepes pH 7.2, 5 mmol/liter MgCl2, 5 mmol/liter EDTA, 5 mmol/liter dithiothreitol, supplemented with Complete® protease inhibitor mixture (Roche Diagnostics) and lysed by 4 freeze/thaw cycles. After centrifugation at 14,000 × g for 30 min at 4 °C, protein concentrations and caspase-3 activities were determined in the supernatant according to the protocol of the CaspACE™ Fluorimetric Assay System (Promega). Data are expressed as picomole of the fluorescent substrate liberated per minute and microgram of protein.

TaqMan™ Real Time Quantitative RT-PCR—Total RNA was isolated with the RNAeasy kit (Qiagen, Hilden, Germany) from VSMCs incubated for different time periods with 100 µM phytanic or pristanic acid alone or in the presence of 1 ng/ml TNFα or 3 ng/ml TGFβ1, respectively. Quantitation of the iNOS mRNA was done by exploiting the 5’ nucleic acid activity of the TaqDNA polymerase to cleave
the indicated time periods. Subsequently, caspase-3 activity was measured in cell extracts as described under “Materials and Methods.” Data represent means ± S.D. of three independent experiments each performed in triplicate.

Subconfluent VSMCs were treated with 100 μM phytanic acid or 1% Me₂SO, and caspase-3 activity was measured in cell extracts as described under “Materials and Methods.” Data represent means ± S.D. of three independent experiments each performed in triplicate.

Materials and Methods.

Effect of fatty acids and PPARα agonists on caspase-3 activity in VSMCs. Subconfluent rat VSMCs were treated with 100 μM of the indicated compounds for 6 h (A) or 100 μM pristanic acid for the indicated time periods. Subsequently, caspase-3 activity was measured in cell extracts. 6 h of incubation with 1% Me₂SO served as control. Data represent means ± S.D. of three independent experiments each performed in triplicate.

A target gene specific TaqMan probe during RT-PCR. All TaqMan probes contained a 6-carboxyfluorescein moiety as reporter dye at the 5’-end and a carboxytetramethyl rhodamine moiety as quencher dye at the 3’-end of the probe. Amplifications and sequence detections were carried out on an ABI PRISM 7700 (PE Applied Biosystems, Weiterstadt, Germany). Primers and TaqMan probes were purchased from MWG Biotech (Ebersberg, Germany). Forward and reverse PCR primers were used at a final concentration of 300 nM, TaqMan probes at a final concentration of 150 nM. Relative expression of the iNOS mRNA were evaluated relative to the expression of the β-actin mRNA in the same sample. All TaqMan experiments were performed using the Universal Master Mix system from PE Applied Biosystems. Cycling parameters were: 1 min 50 °C, 1 min 95 °C, 1 min 60 °C. To prevent amplification of target sequences from contaminating genomic DNA, all RNA samples were subjected to digestion with RNase-free DNase I prior to the RT-PCR step.

Western Blotting of Inducible NOS—Subconfluent VSMCs were treated with 100 μM phytanic or pristanic acid alone or in combination with 3 ng/ml TGFβ1 or 1 ng/ml TNFα antibody for various time intervals. Cells were then washed twice with ice-cold phosphate-buffered saline and harvested in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 25 mM NaF, 0.5% sodium deoxycholate, 10% SDS, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 10 mM sodium pyrophosphate supplemented with Complete® protease inhibitor mixture. Thereafter, the cells were lysed by four freeze/thaw cycles and centrifuged in an Eppendorf benchtop centrifuge for 10 min at maximum speed. 50 μg of supernatant protein were then subjected to Western blotting, performed according to standard procedures. The blots were developed with a 1:4000 dilution of a mouse monoclonal antibody specific for murine iNOS purchased from BD Transduction Laboratories (Lexington, KY).

Reporter Gene Studies—Subconfluent rat VSMCs were grown in standard 6-well dishes and co-transfected with 0.5 μg/well of a pCAT3 vector (Promega) containing the NFκB response element and the pCMV-βgal vector together with 1.5 μg per well of FuGENE 6 transfection reagent (Roche Diagnostics). 20 h after transfection, the cells were incubated in Dulbecco’s modified Eagle’s medium containing 1% fetal calf serum and 100 μM phytanic or pristanic acid for various time intervals. β-galactosidase and CAT activities were measured with an ELISA detection kit supplied by Roche Diagnostics.

TNFα ELISA—Subconfluent rat VSMCs were treated with 100 μM phytanic or pristanic acid for the indicated time intervals, and the TNFα concentration was determined in the culture medium employing a commercially available ELISA kit for rat TNFα (Biozol, Eching, Germany). The lower detection limit of the assay was ~17 pg/ml. For each experiment, a standard curve was obtained by serial dilutions of a rat TNFα stock solution to yield 0–500 pg/ml.

RESULTS

To evaluate the effect of phytanic acid on exponentially growing rat VSMCs, 70% confluent cells were incubated with increasing concentrations of phytanic acid in the presence of 1% fetal calf serum as a survival factor. After 4 h of incubation in the presence of 100 μM phytanic acid, cells rounded, shrunk, and lost contact to their neighbors (Fig. 1). As these observations were suggestive of cell death, we next stained VSMCs treated with phytanic acid with YO-PRO in order to detect plasma membrane permeability. As shown in Fig. 2A, the fraction of YO-PRO-positive cells increased at 6 h of incubation with 100 μM phytanic acid compared with the Me₂SO-treated control. Another well characterized marker for the detection of apoptotic and/or necrotic cells consists of annexin V binding (19). As shown in Fig. 2B, annexin V binding increased upon incubation with the fatty acid. Differentiation of apoptosis from necrosis was performed by three means. (i) Simultaneous incubation with propidium iodide and fluorescently labeled annexin V showed that >90% of the cells were annexin V-positive/propidium iodide-negative. (ii) We induced necrotic cell death with a 5-min incubation at 42 °C and compared the propidium iodide staining of the necrotic cells with that of the BCFA-treated cells by flow cytometry. Based on the fluorescence intensity per cell, we identified a fraction of only 5.6% of necrotic cells after 6 h of treatment with 100 μM phytanic acid (control: 4.0%) (data not shown). (iii) We measured the activity of

FIG. 3. Effect of phytic acid on caspase-3 activity in VSMCs from rat, human, and porcine origin. Subconfluent VSMCs were incubated for 6 h with 100 μM phytic acid or 1% Me₂SO, and caspase-3 activity was measured in cell extracts as described under “Materials and Methods.” Data represent means ± S.D. of three independent experiments each performed in triplicate.

FIG. 4. Effect of fatty acids and PPARα agonists on caspase-3 activity in VSMCs. Subconfluent rat VSMCs were treated with 100 μM of the indicated compounds for 6 h (A) or 100 μM pristanic acid for the indicated time periods. Subsequently, caspase-3 activity was measured in cell extracts, 6 h of incubation with 1% Me₂SO served as control. Data represent means ± S.D. of three independent experiments each performed in triplicate.

FIG. 5. Effect of iNOS inhibitors on phytic acid induced apoptosis. VSMCs were incubated with 100 μM phytic acid for 6 h in the presence of the artificial NOS inhibitors, monomethyl-L-arginine monosuccinate (L-NMMA, 250 μM) or aminohydroxyguanidine (AHG, 200 μM) or TGFβ1 (3 ng/ml). Cells treated with Me₂SO (1%) served as controls. Caspase-3 activity was measured in cell extracts as described under “Materials and Methods.” Data represent means ± S.D. of three independent experiments each performed in triplicate.
caspase-3, which is a major effector during the execution phase of apoptosis (20). We found that phytanic acid led to a steep time and dose-dependent increase in caspase-3 activity (Fig. 2C). The maximum effect was observed at a concentration of 100 μM of the fatty acid. To characterize the species specificity of phytanic acid induced apoptosis, we next incubated VSMCs from porcine and human origin with 100 μM of this fatty acid. As is evident from Fig. 3, the effect of phytanic acid was not species-specific, although human cells were somewhat less susceptible in comparison to rat and porcine ones. Neither human umbilical vein endothelial cells nor human monocyte-derived macrophages could be forced to programmed cell death by a 6-h incubation with 100 μM phytanic acid (data not shown).

Since phytanic acid represents a potent activator of PPARα (2–4), we next investigated whether it shared its pro-apoptotic property with other known natural or artificial PPARα agonists. We incubated rat VSMCs with palmitic acid, nervonic acid or linoleic acid, as well as with the artificial activators bezafibrate and Wy 14,643. None of these compounds were able to induce morphological changes, which were indicative for apoptosis (not shown). They also did not increase caspase-3 activity when administered to the cells for 6 h at concentrations up to 250 μM (data not shown). In vivo, phytanic acid is degraded via an α-oxidation step, yielding pristanic acid, before entering the peroxisomal β-oxidation cycle (21). Pristanic acid activates PPARs more effectively than phytanic acid but it does not activate RXR (4, 10). Therefore, we examined the effect of pristanic acid on VSMC apoptosis. We found that pristanic acid at a concentration of 100 μM for 6 h led to a significant activation of caspase-3 (Fig. 4A). The time course of caspase-3 activation differed between the two BCFA. At 100 μM, maximal caspase-3 activity was reached already between 2 and 3 h compared with 5 h for phytanic acid (Fig. 4B).

To elucidate the mechanism of phytanic- and pristanic acid-induced apoptosis we characterized key regulators of the process. Since induction of apoptosis in VSMCs can be mediated by the production of NO (22, 35), we reasoned that the BCFA effect might be due to excessive production of this compound. Since NO is produced via nitric-oxide synthases, we co-incubated VSMCs with phytanic or pristanic acid and different NOS inhibitors to test this hypothesis. We could demonstrate that the competitive inhibitor of all three NOS isoforms, monomethyl-L-arginine monoacetate (L-NMMA, 250 μM), as well as addition of the specific inducible NOS inhibitor, aminohydroxyguanodine (AHG, 200 μM), prevent BCFA induced apoptosis (Fig. 5). Our hypothesis that BCFA-induced apoptosis is mediated by NO was furthermore consistent with the observation that VSMCs treated simultaneously with phytanic

**Fig. 6. Induction of iNOS after treatment with phytanic acid.** A, rat VSMCs were treated with 100 μM phytanic acid alone and in combination with 3 ng/ml TGFβ1 for the indicated time periods. The amount of iNOS-specific mRNA was quantified using real-time PCR as described under “Materials and Methods.” Data represent means ± S.D. of three independent experiments. B, VSMCs were incubated in the presence of Me2SO (1%), phytanic acid (100 μM), and TGFβ1 (3 ng) for 4 h. The cell extracts were subjected to Western blot analysis. The blots were developed with a monoclonal antibody specific for rat iNOS as described under “Materials and Methods.”
Phytic Acid-induced VSMC Apoptosis

Fig. 7. Time course of phytic acid induced NFκB activation and inhibition of apoptosis by blocking TNFα with a neutralizing antibody. A, subconfluent rat VSMCs were transfected for 20 h with a plasmid containing the responsive element for NFκB upstream of a CAT reporter gene as well as with a β-gal normalization vector as described under “Materials and Methods.” 48 h after transfection, the cells were incubated with 100 μM phytic acid for the indicated time periods. Subsequently, β-galactosidase and CAT activities were measured, and relative NFκB activities were calculated. Data represent means ± S.D. of three independent experiments. B, rat VSMCs were incubated with phytic acid (100 μM) for 6 h alone or together with a TNFα-neutralizing antibody (1 ng/ml). Caspase-3 activity was measured in cell extracts as described under “Materials and Methods.” Cells treated with 1% Me2SO or 1 ng/ml of the TNFα-neutralizing antibody in the absence of phytic acid served as controls. Data represent means ± S.D. of three independent experiments each performed in triplicate.

acid and TGFβ1 were partially protected from programmed cell death (Fig. 5). It is known that TGFβ1 destabilizes iNOS mRNA, which decreases the amount of NO production (23). As regulation of NO release from iNOS-expressing cells occurs predominantly at the DNA transcription and/or protein stability level we next analyzed the expression pattern of iNOS by quantitative RT-PCR and Western blot analysis. Cultured VSMCs were treated for different time intervals with 100 μM phytic acid alone or in combination with 3 ng/ml of TGFβ1 (Fig. 6). We could demonstrate that the amount of iNOS mRNA and protein markedly increased 2 h after onset of incubation and increased further with the duration of treatment with phytic acid (Fig. 6, A and B). Co-incubation with TGFβ1 inhibited the increase in iNOS expression significantly at the level of mRNA (Fig. 6A) and also of iNOS protein (Fig. 6B).

Since the promoter region of the iNOS gene includes several binding sites for nuclear factor κB (NFκB), we next measured NFκB activity using a transactivation assay. Subconfluent VSMCs were transfected with a plasmid containing a CAT reporter gene under the control of the NFκB responsive element as well as with a β-gal normalization vector. 20 h after transfection cells were incubated with 100 μM phytic acid for different time periods and CAT and β-galactosidase activities were measured. NFκB was activated shortly (between 15 and 30 min) after addition of phytic acid, and its activity peaked at 60 min of incubation (Fig. 7A).

TNFα, a membrane-bound cytokine that is cleaved by a specific membrane metalloprotease upon activation, is a well known activator of iNOS. Therefore, we next analyzed whether this cytokine may be involved as upstream factor in phytic acid-induced apoptosis. To study the involvement of TNFα, we first inhibited the mature soluble form of TNFα in the medium by incubating VSMCs simultaneously with 100 μM phytic acid and a TNFα neutralizing antibody. As shown in Fig. 7A, blocking TNFα with the antibody prevented the phytic acid-mediated activation of the NFκB reporter gene construct almost completely. Moreover, the pro-apoptotic potential of phytic acid resulting in increased caspase-3 activation was also almost completely abolished in the presence of the TNFα antibody (Fig. 7B), whereas incubation with a series of IgG fractions obtained from control sera had no effect (data not shown). To further corroborate this data we investigated whether the TNFα antibody was capable of preventing the intensive phytic acid-induced expression of iNOS. VSMCs were treated with 100 μM phytic acid in the presence or absence of 1 ng/ml of the TNFα-antibody. As shown in Fig. 8, A and B, the TNFα antibody reduced the level of iNOS protein, as detected in Western blots of cell extracts, and the steady state concentrations of the iNOS mRNA in comparison to VSMCs incubated with phytic acid alone. To show the role of TNFα in a more direct manner, we next quantified the amount of functional TNFα in the medium. Subconfluent VSMCs were incubated with 100 μM phytic acid for different time intervals between 30 min and 3 h. Subsequently, the concentration of mature TNFα was measured in the medium using a rat TNFα-specific ELISA. As shown in Fig. 8C, the content of TNFα in the medium was significantly higher in those media obtained from cells that had been treated with phytic acid compared with the non-treated controls.

Since these data suggest that TNFα induction may be an upstream event involved in apoptosis induction by BCFA, we continued to investigate whether TNFα alone is sufficient of induce apoptosis in VSMCs. The cells were incubated in the presence of TNFα with or without cycloheximide for 8 h and caspase-3 activity was measured. As shown in Fig. 9, TNFα did not induce caspase-3 activation at concentrations of 20 or 40 ng/ml. In contrast, a moderate activation could be observed in the presence of TNFα and cycloheximide. However, the effect was distinctively smaller than obtained with phytic acid or pristanic acid.

DISCUSSION

In this study, we show that BCFA induced apoptosis in VSMCs and that they exerted their effect via TNFα secretion and iNOS up-regulation. This is the first time that a fatty acid has been reported to induce apoptosis in VSMCs in vitro. However, it is known that various straight-chain fatty acids, like palmitate and stearate, are able to induce apoptosis in other cell types, such as cardiac myocytes or pancreatic β cells (24–26). Under the conditions of our study, the pro-apoptotic effect was specific for isoprenoid BCFA like phytanic and pristanic acid and was not mimicked by saturated or unsaturated straight chain fatty acids. To test whether albumin affected the apoptosis-promoting activity of BCFA, we also performed the assays with media supplemented with up to 30 mg/ml of fatty acid-free bovine serum albumin (which would be sufficient to bind ~3 μM of fatty acid assuming a binding stoichiometry of 7:1). However, albumin neither inhibited the apoptosis promoting effect nor altered its specificity (data not shown). VSMCs of porcine and rat origin showed similar susceptibility toward BCFA-induced apoptosis whereas this effect was somewhat less pronounced in human VSMCs. Whether this difference was due to more rapid degradation of BCFA, the preponderance of other signaling pathways or to more efficient up-regulation of anti-apoptotic survival genes remains to be elucidated. Regarding the major cell types of the vascular wall, the pro-
The apoptotic effect of BCFA was specific for VSMCs as these fatty acids failed to cause programmed cell death in human umbilical vein endothelial cells and human monocyte-derived macrophages if subjected to the same conditions as were employed for VSMCs (data not shown).

Our group and others (2–4) reported previously that phytanic and pristanic acid are activating ligands of PPARα. PPARα was first identified in liver cells as an important regulator of fatty acid metabolism but is also expressed within cells of the vascular wall like endothelial cells, smooth muscle cells and macrophages (6, 8). It was shown that stimulation of PPARα with the hypolipidmic drug Wy 14,643 causes apoptosis in macrophages (8) whereas it suppresses the IL-1β-induced inflammatory response in human VSMCs (7). In this study, we demonstrate a pro-apoptotic effect of phytanic and pristanic acid, which is neither shared by the very potent artificial PPARα activator Wy 14,643 nor by several straight chain fatty acids, which have been shown to act as natural PPARα agonists. These findings correspond to results from the group of Staels who demonstrated that an incubation of human VSMCs with high concentrations of Wy 14,643 did not alter the viability of the cells (7). Conclusively, activation of PPARα is most likely not responsible for the programmed cell death caused by BCFA in VSMCs.

Our study reveals some mechanistic insights into BCFA-induced apoptosis. We could demonstrate that apoptosis induced by BCFA depends on TNFα activation and secretion because a TNFα neutralizing antibody blocked apoptosis almost completely. In addition, we could show that BCFA induced a steep increase of TNFα concentrations in the medium of VSMCs shortly after incubation with BCFA. This implies that BCFA or one of their downstream metabolites stimulate the cleavage of the membrane-bound precursor form of TNFα, leading to release of the mature soluble form of the cytokine. In addition, our data support that BCFA, either directly or indirectly, trigger the secretion of TNFα in an autocrine manner, subsequently leading to programmed cell death. Consistent with previous reports (27), we could show however, that TNFα

**Fig. 8.** TNFα is a key regulator of phytanic acid-induced apoptosis in VSMCs. A, rat VSMCs were incubated in the presence of MeSO (1%), phytanic acid (100 μM) alone, or together with a TNFα antibody (1 ng/ml) for 4 h. Subsequently, cell extracts were prepared and subjected to Western blot analysis. The blots were developed with a monoclonal antibody specific for rat iNOS as described under “Materials and Methods.” B, rat VSMCs were treated with 100 μM phytanic acid, either alone or in combination with the TNFα antibody (1 ng/ml) for up to 4 h. The amount of iNOS-specific mRNA was quantified using real-time PCR as described under “Materials and Methods.” Data represent means ± S.D. of three independent experiments. C, time course of mature TNFα concentration in media collected from VSMCs after treatment with 100 μM phytanic acid, MeSO, or medium alone (control) for various time periods. TNFα concentrations were quantified by ELISA and data represent means ± S.D. of six independent experiments.
alone is not sufficient to induce apoptosis in VSMCs. Thus, induction of apoptosis may require TNFα along with a combination of other metabolic changes or cytokines. So far, our attempts to identify the relevant factors that are affected by BCFA along with TNFα and thus may act in concert to induce apoptosis in VSMCs were however not successful.

There are several pathways that are known to lead from TNFα binding to its receptor to programmed cell death. The most prominent is activation of caspase-8 subsequently resulting in activation of effector caspases mediating the key processes during programmed cell death (29). We could demonstrate that caspase-8 is not involved in BCFA-induced apoptosis as no increase of activity of this enzyme could be detected after incubating VSMCs with these fatty acids for various time periods (data not shown). In addition to the signaling pathway leading to caspase-8 activation, TNFα regulates gene expression via activation of several transcription factors. One example for this is the induction of iNOS gene expression as a consequence of cytokine treatment in VSMCs (29). In line with this concept, we could demonstrate that iNOS mRNA as well as protein levels are more than 100-fold up-regulated after incubation of VSMCs with BCFA. The induction of iNOS was obliterated when TNFα secretion was blocked by adding a TNFα-specific antibody to the cells. Furthermore, we were able to show that stimulation of iNOS expression is a necessary step during BCFA-induced cell death as different inhibitors of NO abolished the pro-apoptotic effect of these fatty acids. Also the effects of TGFβ1, previously shown to destabilize iNOS mRNA (30), are consistent with an important role of NO in VSMC apoptosis. Nishio et al. (22) showed that incubation of VSMCs with various NO donors have the ability to induce apoptosis within this cell type (22). So far, it is not entirely clear, however, whether iNOS induction is sufficient to induce apoptosis in VSMCs.

Because we could show directly that iNOS stimulation depends on TNFα secretion, one may speculate that the activation of the first is mediated via NFκB. It has been demonstrated by several groups that iNOS gene expression is tightly regulated by NFκB with several responsive elements being found in the promoter region of the iNOS gene. Accordingly, we observed a significant 2-fold activation of endogenous NFκB when VSMCs were incubated with BCFA. However, it is not clear at present whether a 2-fold up-regulation of the transcription factor may be sufficient to account for the observed more than a hundred-fold induction of iNOS. Moreover, it was demonstrated earlier that NFκB may not be the only transcription factor that can mediate the cytokine-induced activation of iNOS gene expression (31, 32).

Although the conditions of our current in vitro study differ from the situation in the intact organism, we will be interested to study whether apoptosis may be responsible for some of the toxic effects that result from supraphysiological concentrations of BCFA under in vivo conditions. Serum concentrations of phytic acid up to 1 µM are present in patients suffering from peroxiasomal disorders like Zellweger syndrome or Refsum disease. In addition, other conditions are known which are associated with an accumulation of phytic acid along with pristanic acid as a result of a block in the peroxiasomal β-oxidation of BCFA-CoA.