Apoptosis-inducing natural products found *in utero* during murine pregnancy

Stephen D Liberles and Stuart L Schreiber

Background: Hormones, lipids, vitamins and other biologically active small molecules can be removed from animal tissues by extraction with organic solvents. These compounds can have dramatic effects on cultured cells and the characterization of such compounds can lead to the discovery of new functions for known molecules, or even to the discovery of previously unknown compounds.

Results: Organic-soluble compounds in 17.5-day-old mouse embryos were removed with *tert*-butylmethylether and found to induce apoptosis in T-antigen-transformed Jurkat T cells. These embryonic extracts were fractionated and their apoptosis-inducing components were identified as a mixture of polyunsaturated fatty acids, including arachidonic, docosatetraenoic and docosahexaenoic acids. Docosatetraenoic acid was the most potent apoptosis inducer with an effective dose (ED_{50}) of 30 μM.

Conclusions: A family of polyunsaturated fatty acids is shown to be abundant *in utero* during pregnancy. Members of this family are able to induce cleavage of poly(ADP)ribose polymerase, and ultimately to induce apoptosis, in T-antigen-transformed Jurkat T cells. Free radical scavengers, including phenol and benzyl alcohol, block the apoptosis-inducing properties of these polyunsaturated fatty acids; this is consistent with a lipid peroxidation mechanism involving formation of hydroperoxy fatty acids.

Address: Howard Hughes Medical Institute, Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA.

Correspondence: Stuart L Schreiber
E-mail: sls@slsiris.harvard.edu

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While studying the actions of small-molecule dimerizers, we measured the tissue distribution of rapamycin following its injection into transgenic mice. We removed various mouse tissues and extracted them with organic solvent. The presence of rapamycin in these tissue extracts was determined by monitoring their effects on cells engineered to have a transcriptional response to rapamycin [1–3]. We were surprised to observe that the organic extracts of embryos removed from uninjected pregnant mice were able to induce apoptosis in T-antigen-transformed Jurkat T cells (hereafter referred to as TAg Jurkat T cells [2]). The organic extracts of mouse embryos contain a large number of structurally interesting and biologically active compounds and we sought to identify the active apoptosis-inducing component in this mixture.

The concept of studying the molecules that compose animal tissue dates back to the earliest stages of biochemistry research. The isolation of urea from urine by Hermann Boerhavve (1668–1738) and later the synthesis of urea by Friedrich Wohler (in 1828) provided the first well-documented studies of a compound derived from living tissue, thus proving the notion that living systems were tractable to scientific investigation [4]. These results initiated a cascade of research probing the composition of animal tissue, and thus effective procedures fatty acids, carbohydrates, peptides, and later DNA [4].

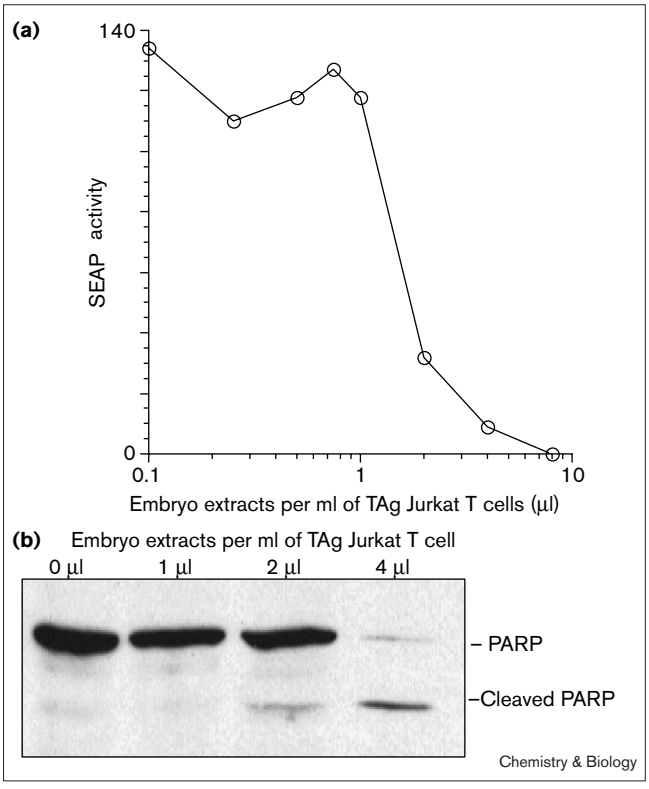
As more was learned about the components of animal tissue, many natural product and synthetic chemists moved away from the study of animal-based small molecules. Instead, they shifted towards the study of small molecules derived from alternative sources, such as microbes, plants and marine organisms, that alter the normal function of animal tissue. Our understanding of biological systems has evolved since the initial isolation of animal-derived small molecules, however, and tissue extracts can now be screened for novel activities that were not previously known or understood at a molecular level. In this study, we describe the production of a natural product mixture from mouse embryonic tissue. Compounds in these embryonic extracts have effects on cells in culture and we report the purification and structural characterization of animal compounds that induce apoptosis in TAg Jurkat T cells.

Results

Induction of apoptosis by crude organic extracts of mouse embryonic tissue

Several tissues, including the uterine segment consisting of both maternal and embryonic tissue, were removed from 17.5 days post-coitum pregnant mice. The dissected tissues were incubated overnight in proteinase K buffer

Figure 1



Induction of apoptosis by crude embryonic extracts. **(a)** Inhibition of a constitutive reporter gene, secreted alkaline phosphatase (SEAP), by embryonic extracts. TAg Jurkat T cells were transfected with a plasmid containing a SEAP reporter construct under control of the nuclear factor of activated T cells (NFAT) enhancer. These cells were then incubated simultaneously with phorbol myristate acetate (PMA), ionomycin and various concentrations of embryonic extracts. After 24 h, SEAP activity was determined as described in the Materials and methods section. **(b)** Poly(ADP)ribose polymerase (PARP) cleavage by embryonic extracts. Wild-type TAg Jurkat T cells were incubated with various concentrations of embryonic extracts for 5 h. Samples were lysed and analyzed by western blot using an antibody that recognizes PARP.

and extracted with *tert*-butylmethylether. The organic layers were then dried, washed and resuspended in ethanol to prepare them for tissue culture experiments.

The organic extracts of a number of tissues, including uterine, brain, and liver tissue, can kill TAg Jurkat T cells; this was initially observed by the inhibition of a constitutively expressed enzymatic reporter, secreted alkaline phosphatase (SEAP). The organic extracts of mouse embryos were found to be the most potent. Incubation of 1 ml of TAg Jurkat T cells (1×10^6 cells/ml) with 2–5 µl of embryonic extracts, representing the organic compounds from about 100 µl of embryonic tissue, is sufficient to induce cell death (Figure 1a).

To test whether the organic extracts of mouse embryonic tissue induce apoptosis, we examined whether poly(ADP)ribose polymerase (PARP) is cleaved after treatment with extract. PARP is a DNA repair enzyme that is rapidly cleaved during apoptosis, presumably so that it cannot counteract the nucleases that ultimately kill the cells [5]. Conversion of PARP from the full-length 116 kDa protein to the inactive 85 kDa fragment is observed within 5 hours of treatment with embryonic extracts (Figure 1b). The minimal amount of embryonic extracts required to induce PARP cleavage is close to the IC_{50} for SEAP inhibition. At higher concentrations of embryo extracts, neither full-length PARP nor the 85 kDa cleavage product could be detected, perhaps because at these high concentrations a constituent of embryo extracts induces necrotic, rather than apoptotic, cell death.

Purification of the apoptotic activity from mouse embryos

A two-step purification process was used to isolate the substance having apoptotic activity from mouse embryos. During the first step the organic extracts from two to three litters of embryos (corresponding to about 20–30 mouse embryos) were separated on silica gel using a solvent gradient of increasing polarity. The active fractions eluted off the column slowly with 25% ethyl acetate/hexane, and all subsequent columns were run using only this solvent mixture as the mobile phase. The diversity of compounds in the embryonic extracts can be seen on a thin-layer chromatogram of the silica gel column fractions (Figure 2a). Fractions with apoptotic activity were pooled.

Analysis of the pooled active fractions by mass spectrometry (Figure 2b) revealed a family of co-migrating molecules. Two trends were observed in this mass spectrum. First, the major compound peaks differed from each other by 22–28 atomic mass units, where 28 is the molecular weight of an ethylene ($–CH_2CH_2–$) unit. Second, each major compound peak was surrounded by minor compound peaks that differed in mass by exactly two or multiples of two, thus suggesting the presence or absence of double bonds. An explanation for these observations is shown in Figure 2c. A second family of peaks was also observed in the higher mass end of the spectrum. These peaks could correspond to dimers of the lower molecular-weight peaks, or to an entirely different family of molecules.

As a number of compounds co-migrated on the silica-gel column, a second purification step was required to isolate the active compounds. Recycling high performance liquid chromatography (recycling HPLC), using columns that resolve compounds by size and polarity, separated the mixture further and the apoptosis-inducing activity co-migrated with one of two closely running fractions. The mass spectra of these fractions (Figure 3a) suggested improved purity as only a subset of the original peaks was present in each fraction. The results also suggested that the higher molecular-weight peaks correspond to dimers

of the lower molecular-weight species. An IR spectrum was obtained for each of these fractions (data not shown). The data demonstrated that the compounds are both similar and structurally simple. All of the compounds contain only one stretch in the carbonyl region of the spectrum; the only interpretable difference between the IR spectra of these compounds is the amplitude of the stretch at 3010 wave numbers, suggesting a difference in the number of double bonds in these molecules. One inactive peak, corresponding to a molecular weight of 282, was remarkably pure. Structural characterization of the compound in this purest fraction, although it was inactive, would provide structural information about the closely migrating active peak.

Analysis of this compound by gas chromatography and mass spectrometry (GC/MS) led to its identification as oleic acid, based on its fragmentation pattern in the mass spectrum. Likewise, the IR spectrum of oleic acid matched identically with this compound (Figure 3b). Because the other three compounds differ from oleic acid only by the number of carbons and double bonds, as suggested by both MS and IR, we assigned the other three peaks as arachidonic, docosatetraenoic and docosahexaenoic acids. The structures of these compounds are shown in Figure 3c.

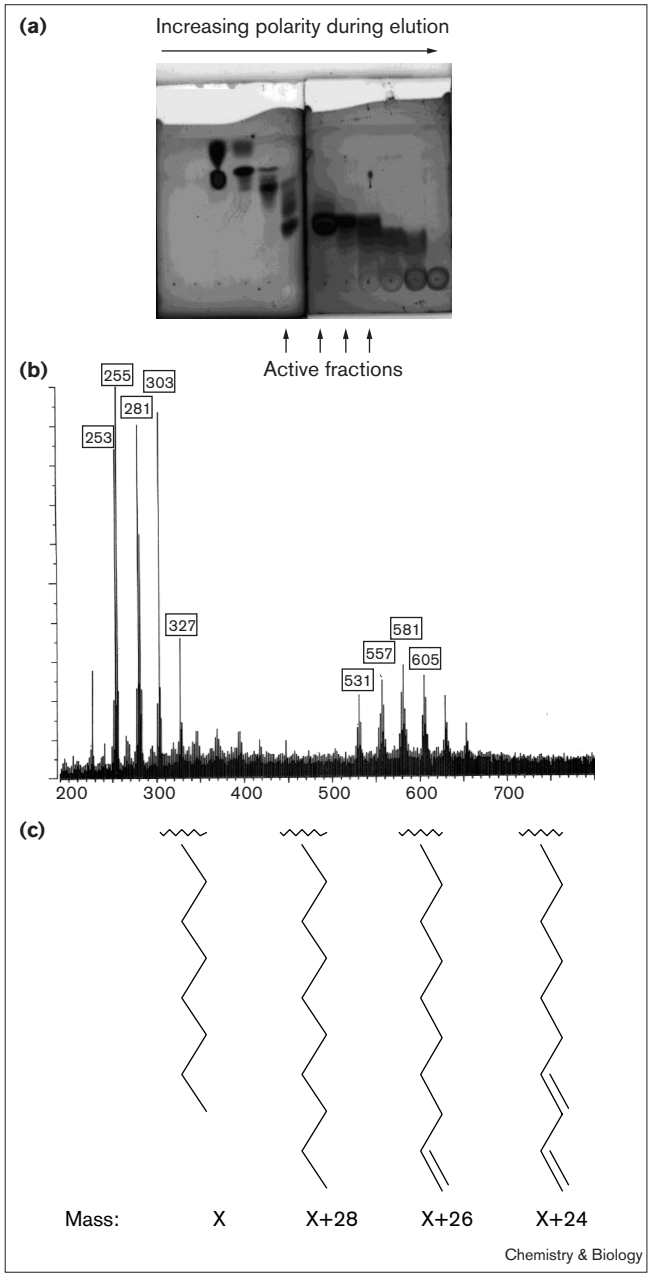
Effects of authentic polyunsaturated fatty acids on TAg Jurkat T cells

To verify that these polyunsaturated fatty acids are indeed the apoptosis-inducing molecules from mouse embryos, authentic samples of arachidonic, docosahexaenoic and docosatetraenoic acids were obtained and compared in the apoptosis assays. All three polyunsaturated fatty acids were found to be toxic to TAg Jurkat T cells, as determined by inhibition of the activity of a constitutive reporter gene product (Figure 4a). Both arachidonic and docosahexaenoic are similarly potent, with an ED_{50} of about 70 μM; docosatetraenoic acid is slightly more potent with an ED_{50} of 30 μM.

The polyunsaturated fatty acids also induce cleavage of PARP, with docosatetraenoic acid being active at the lowest concentration, between 30–70 μM (Figure 4b). The minimal concentrations of docosahexaenoic acid and arachidonic acid required to cleave PARP are 70–100 μM and 200 μM, respectively. Incubating cells with about 300 μM concentration of any of the fatty acids results in the complete disappearance of full-length or cleaved PARP as judged by western blot analysis, a result similar to that induced by high concentrations of crude embryo extracts.

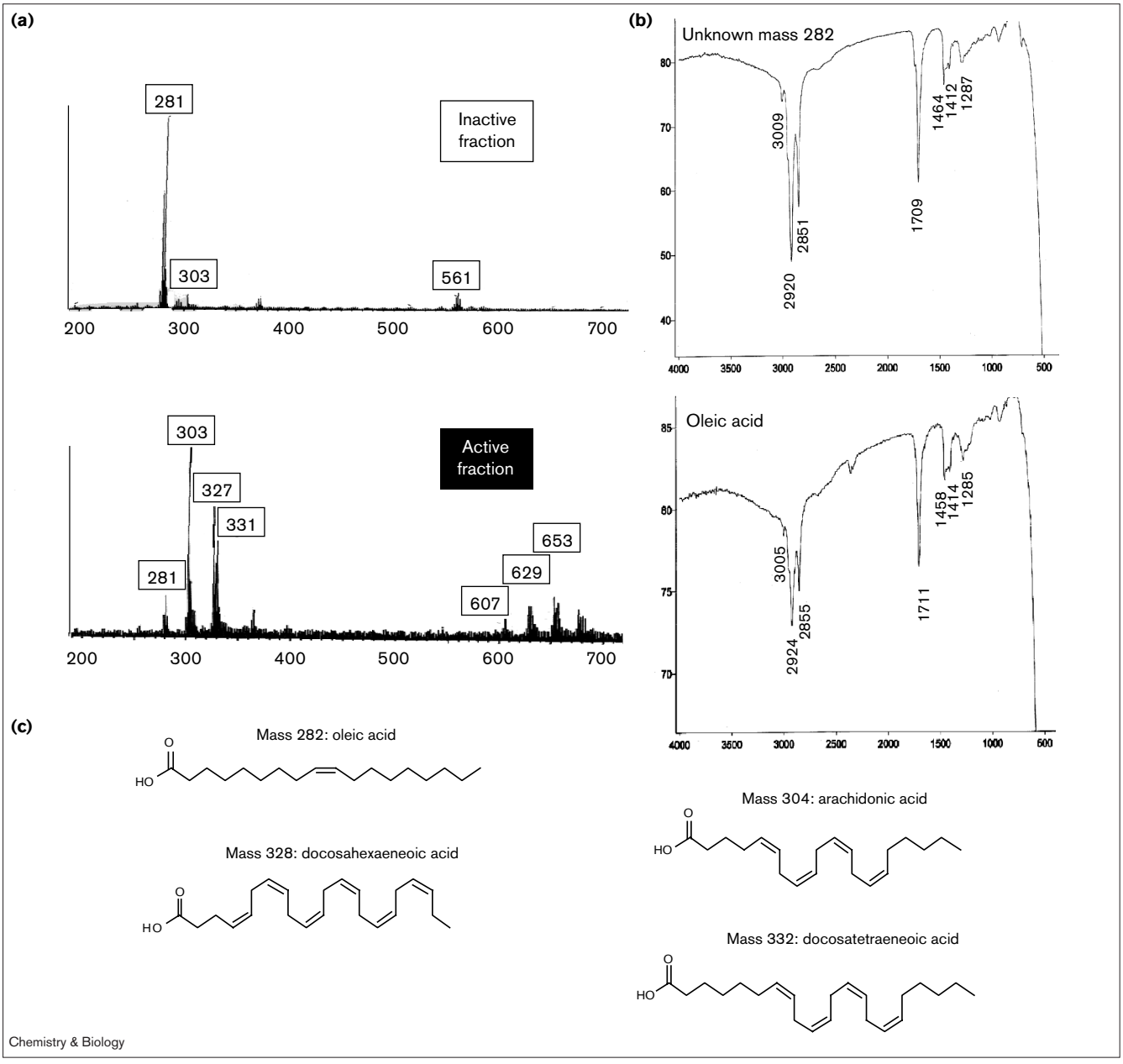
On the basis of these inhibitory constants, we can estimate the concentration at which these compounds exist in the mouse embryo. Extraction of the organic-soluble

Figure 2



Partial purification of the apoptosis-inducing constituent by silica gel chromatography. **(a)** Thin-layer chromatogram of compounds from mouse embryos. The components of mouse embryo extracts were fractionated on silica gel and a thin-layer chromatogram was performed on the fractions using 50% ethyl acetate/hexane as a mobile phase. Those fractions with apoptotic activity are indicated. **(b)** Mass spectrum of silica-gel-purified embryonic extracts. The active fractions shown in (a) were pooled and mass spectrometry was performed on an aliquot of the pooled fractions. In the mass spectrum, two families of peaks were observed, and the members of each family differed in mass by 22–28 atomic mass units. Furthermore, each peak was surrounded by minor peaks that differed in mass by two or multiples of two. **(c)** An explanation for how two structurally similar compounds could differ in mass by 24, 26 or 28 atomic mass units.

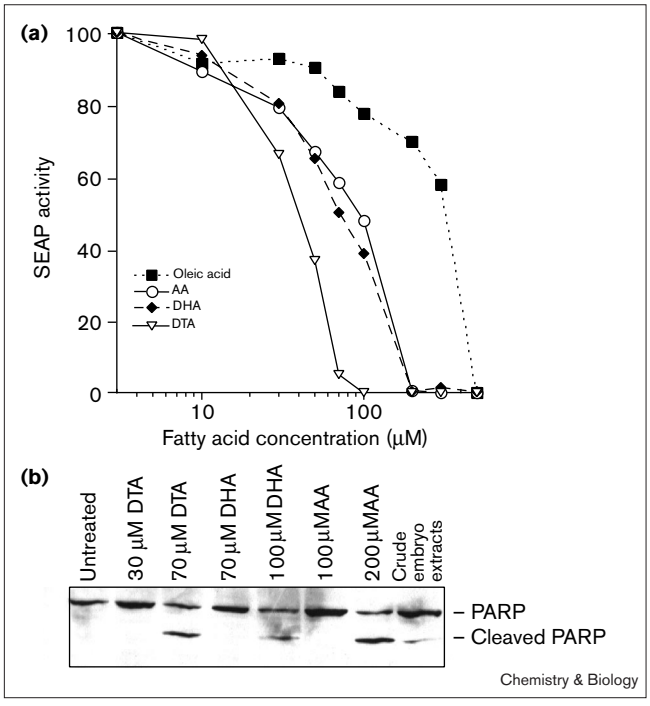
Figure 3



Structural determination of the active apoptosis-inducing constituent. **(a)** Mass spectra of two closely migrating JAI peaks. The pooled silica-gel-purified material was injected on the JAI recycling HPLC. The fraction displaying apoptotic activity had only a subset of the compounds found in the pre-JAI-purified material. Furthermore, a closely migrating inactive compound was pure. **(b)** Comparison of the inactive peak to oleic acid using infrared (IR) spectroscopy. After gas chromatography and mass spectrometry (GC/MS) identified the inactive peak as oleic acid, this assignment was confirmed by comparison of the IR spectra of these compounds. As the IR spectra matched closely, we judged the 282 molecular-weight peak to be oleic acid. **(c)** Structures of the mouse embryonic lipids found in the active fraction of the JAI-purified material.

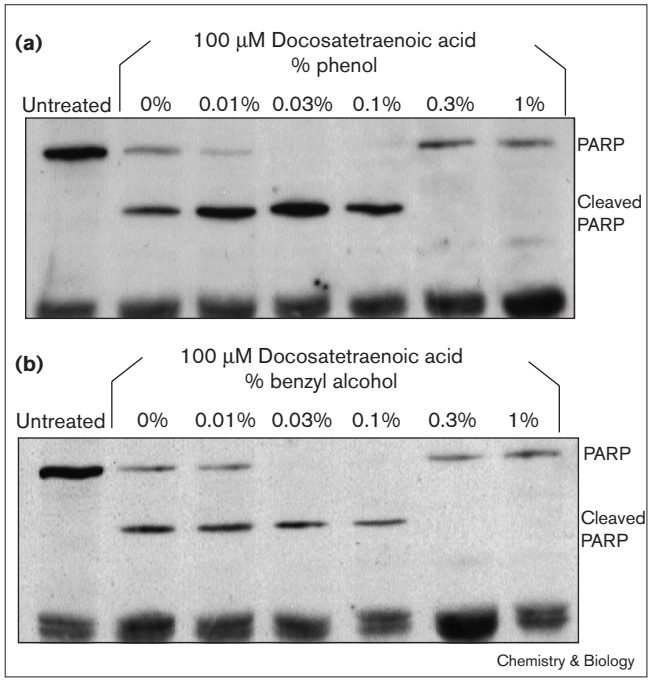
constituents of about 200 µl of embryonic tissue provides the minimal amount sufficient to induce apoptosis in 1 ml of TAg Jurkat T cells. As the IC_{50} for apoptosis by these polyunsaturated fatty acids is between 30 and 100 µM, the original concentration is ~150–500 µM in mouse embryos, assuming complete recovery of these compounds during the extraction process. This calculation also assumes that all of the apoptotic activity in the extracts is due to the polyunsaturated fatty acids. During the silica-gel fractionation, a second minor peak of apoptotic activity elutes

Figure 4



Authentic polyunsaturated fatty acids induce apoptosis in TAg Jurkat T cells. **(a)** Inhibition of SEAP activity by authentic samples of unsaturated fatty acids. TAg Jurkat T cells were transfected with NFAT-SEAP, and treated simultaneously with PMA, ionomycin and various concentrations of fatty acids, as indicated. After 24 h, SEAP assays were performed as described in the Materials and methods section. **(b)** Cleavage of PARP induced by polyunsaturated fatty acids. TAg Jurkat T cells were incubated for 5 h with the indicated concentrations of polyunsaturated fatty acids. DTA, docosatetraenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid. Cleavage of PARP was determined by western blot analysis.

Figure 5



Suppression of docosatetraenoic-acid-induced PARP cleavage by phenol and benzyl alcohol. TAg Jurkat T cells were incubated with the indicated concentrations of **(a)** phenol and **(b)** benzyl alcohol for 10 min and then incubated either alone or with 100 μM docosatetraenoic acid for 5 h. Cleavage of PARP was monitored by western blot analysis.

under more polar solvent conditions, but this peak provides only a small percentage of the total apoptotic activity from the crude extracts.

Anti-oxidants block fatty-acid-induced PARP cleavage

To probe the mechanism of apoptosis induced by these polyunsaturated fatty acids, we tested whether lipoxygenase inhibitors (30 μM nordihydroguaiaretic acid [NDGA] or 1 mM esculetin) or cyclooxygenase inhibitors (1 mM aspirin or 50 μM indomethacin) could block PARP cleavage and SEAP inhibition induced by polyunsaturated fatty acids. None of these compounds suppresses the effects of polyunsaturated fatty acids in either assay. We then tested a panel of presumed anti-oxidants for inhibition of PARP cleavage as a number of such compounds, most notably phenol and benzyl alcohol, can block tumor necrosis factor (TNF)-induced apoptosis in L929 cells [6]. Some anti-oxidants have no effect, such as 50 μM vitamin E, whereas others, including phenol and benzyl alcohol, do cause the inhibition of docosatetraenoic-acid-induced PARP cleavage (Figure 5). The minimal concentration of phenol or benzyl alcohol required to block PARP cleavage induced by 100 μM docosatetraenoic acid is 0.3%, about a 300-fold molar excess. Other anti-oxidants tested, 1% dimethyl sulfoxide (DMSO) and 1.2 mM cysteine, also partially blocked polyunsaturated-fatty-acid-induced PARP cleavage (data not shown). One interpretation of these results is that the mechanism of polyunsaturated fatty-acid-induced PARP cleavage in TAg Jurkat T cells involves oxidation of the lipids, perhaps by reactive oxygen species found in these cells, although the mechanism of phenol and benzyl alcohol protection is yet to be elucidated.

Discussion

Arachidonic, docosatetraenoic and docosahexaenoic acids are members of a well-known family of polyunsaturated fatty acids. These compounds were isolated from mouse embryonic tissue based on their ability to induce apoptosis in TAg Jurkat T cells. Pure samples of these compounds induce apoptosis at concentrations between 30 and 100 μM, whereas their concentration in living embryos appears to be higher, based on the amount of crude organic extract required to induce apoptosis. These fatty acids have been shown previously to have effects on many cell lines, including the ability to induce apoptosis, and their presence *in utero* prior to birth is important for the

pregnancy process and proper embryo development, as shown by deprivation experiments.

Direct addition of these polyunsaturated fatty acids to several cell lines, particularly cancer cell lines [7], can induce apoptosis. For example, polyunsaturated fatty acids can induce apoptosis in pancreatic B-cell lines at a concentration of 1 mM and have been implicated as the causative agent of non-insulin-dependent diabetes mellitus as a result of their apoptosis-inducing properties [8]. Furthermore, many apoptotic stimuli elicit the controlled release of arachidonic acid, including TNF, a cytokine that was initially identified by its ability to inhibit the proliferation of cancerous tissues. Treatment of L929 cells with TNF leads to phospholipase A_2 activation [9,10] and arachidonic acid release [11,12], and blocking phospholipase A_2 activation mitigates the toxicity of TNF [6]. Arachidonic acid has also been implicated in the apoptosis of B and T cells during lymphocyte maturation [13]. Phospholipase A_2 can be activated by stimulation of antigen receptors in immature B and T cells, where antigen receptor stimulation leads to apoptosis. In contrast, phospholipase A_2 is not expressed and arachidonic acid is not released during the activation of mature lymphocytes [13]. These results demonstrate a correlation between the presence and absence of arachidonic acid with a change in the phenotypic outcome of antigen receptor stimulation from a decision to live to a decision to die.

The ability of polyunsaturated fatty acids to induce apoptosis might depend on the presence of reactive oxygen species in the cell [14,15]. Reactive oxygen species, generated in the mitochondria of cells undergoing apoptosis, can initiate a catalytic peroxidation of polyunsaturated lipids [14]. The resulting hydroperoxy fatty acids can induce apoptosis directly in many cell lines [16]. Cells that are normally resistant to polyunsaturated fatty acids can be rendered sensitive to them by expression of a cytochrome that increases intracellular levels of reactive oxygen species [17]. Furthermore, several anti-apoptotic agents reduce the cellular concentration of reactive oxygen species. Bcl-2 is an anti-apoptotic gene product that is localized to the generation sites of radical oxygen species, such as the mitochondria [18]. Overexpression of Bcl-2 lowers the levels of hydroperoxy fatty acids generated following apoptotic stimuli and blocks cell death normally induced by oxidative stress [18]. Oxygen radical scavengers, such as phenol and benzyl alcohol, that can reduce the levels of reactive oxygen species and block TNF-induced cytotoxicity in L929 cells [6] can also block polyunsaturated-fatty-acid-induced apoptosis in TAg Jurkat T cells. One mechanism that is consistent with these observations is that polyunsaturated fatty acids when administered to TAg Jurkat T cells react with a catalytic amount of radical oxygen species to form hydroperoxy fatty acids that promote the death of the cells. Whether polyunsaturated fatty acids undergo similar radical-mediated reactions under physiologically relevant conditions in living embryos is unclear.

The high concentration of the unsaturated fatty acids described in this study in embryonic tissue is known to be essential for proper development. A critical role for polyunsaturated fatty acids in embryos is suggested by a mouse engineered to be deficient in phospholipase A_2 by gene deletion [19,20]. Male phospholipase A_2 'knockout' mice develop and reproduce normally, but female knockout mice are infertile, and almost all of their offspring die before birth. Deficiency of an arachidonic acid metabolite, prostaglandin $F_{2\alpha}$, is proposed to explain the failure of the pregnancy process [19,21] and perhaps the high concentration of polyunsaturated fatty acids we observe *in utero* serves as a reservoir for the synthesis of such key metabolites during pregnancy.

Important roles for fatty acids in early development and pregnancy have also been suggested by dietary studies. As mice and humans cannot make highly unsaturated fatty acids *de novo* [22], they must be made available through their diet; depriving mice of these essential fatty acids has highlighted some of the roles of these lipids in living animals. From such studies, significant amounts of docosahexaenoic acid are required for proper development of the central nervous system and retina [23]. The primary source of docosahexaenoic acid is from either the direct uptake of the lipid itself or from a biosynthetic precursor, linolenic acid, which can be converted into docosahexaenoic acid *in vivo* [22]. Pregnant mice fed a diet deficient in linolenic and docosahexaenoic acids give birth to offspring that have impaired visual and learning activity. In humans, these effects are seen in premature infants fed similar diets [23]. Supplementing sufficient quantities of docosahexaenoic acid into the diet of premature infants is now realized to ensure proper visual and cognitive development of human infants [23].

Other fatty acids, including linoleic and arachidonic acids, are also required in the diet at early stages of life [22], and mice deprived of these fatty acids display a number of defects including stunted growth. Human infants born preterm have diminished growth rates for at least a year compared to infants born at term, and this problem can be mitigated by arachidonic acid supplementation [24]. Thus, it is generally appreciated that sufficient levels of arachidonic acid and docosahexaenoic acid are important in the diet at early stages of life, but the developmental role, if any, of docosatetraenoic acid, the most potent apoptosis-inducer isolated in this study, remains unclear.

Significance

We report that the organic extracts of mouse embryos induce apoptosis in T-antigen-transformed (TAg) Jurkat

T cells. Purification and structural characterization of the active components identified them as a family of polyunsaturated fatty acids, including docosatetraenoic acid, docosahexaenoic acid and arachidonic acid. The apoptosis-inducing properties of these polyunsaturated fatty acids were verified using authentic samples of the compounds. Furthermore, they were sensitive to free-radical inhibitors, consistent with a mechanism involving catalytic lipid peroxidation by reactive oxygen species. The abundance of these compounds *in utero* prior to birth is consistent with the proposed roles of these compounds in the pregnancy process and in early embryo development. Furthermore, the ability of these compounds to induce apoptosis has been implicated in both disease processes, such as non-insulin-dependent diabetes mellitus and ethanol-induced liver toxicity [17], and fundamental natural processes, such as lymphocyte maturation and the antiproliferative effects of tumor necrosis factor (TNF). Further research will clarify both the roles these compounds play in pregnant mothers and developing embryos, and the physiological significance of the apoptosis-inducing properties of these polyunsaturated fatty acids.

Materials and methods

Tissue culture

TAg Jurkat T cells were purchased from American type culture collection (ATCC) and maintained in RPMI media supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glutamine. TAg Jurkat T cells were obtained and grown as previously described [2]. Arachidonic, docosatetraenoic and docosahexaenoic acids were purchased from Sigma. IR spectra were obtained on a Galaxy Series FTIR 3000 and GC/MS data were obtained using Hewlatt Packard instrumentation. Mass spectra were obtained with a JEOL SX-102 by negative ion, fast atom bombardment using glycerol as the matrix.

Preparation of embryo extracts

One litter of 17.5-day-old mouse embryos, including maternal uterine tissue, was removed and incubated in proteinase K buffer overnight at 55°C. (Proteinase K buffer: 100 mM Tris pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg/ml proteinase K.) The samples were extracted twice with 5 ml *tert*-butylmethylether, vortexed and centrifuged at 2600 rpm. The organic layers were removed and dried under a nitrogen stream. The resulting residue was extracted twice with 500 μl ethanol and the ethanol suspensions were transferred to an Eppendorf tube and centrifuged (1 min, 14,000 rpm) to remove ethanol-insoluble material. The ethanol-soluble portion was dried under vacuum. A pellet of about 100 μl was typically obtained from one litter of mouse embryos. These pellets were frozen, and later dissolved in a final volume of 200 μl ethanol for cell-based experiments.

PARP cleavage assays

1 ml TAg Jurkat T cells (1×10^6) were placed in a six-well plate and exposed to a variety of conditions including crude organic extracts or pure lipids for 5 h. The cells were transferred to a 1.5 ml Eppendorf tube and pelleted by centrifugation (1 min, 5000 rpm). The supernatant was discarded and the cells were lysed in 40 μl lysis buffer (10 mM Hepes (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 20 μg/ml leupeptin). After addition of 20 μl 6× sample buffer, the samples were boiled, sonicated, and pelleted (14,000 rpm, 1 min) prior to loading on a 6% SDS polyacrylamide gel. The gels were transferred to Immobilon P and PARP protein was detected by western blot analysis using C2-10 primary antibody to PARP (Clontech), sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP; Amersham) and chemiluminescent detection of HRP (Amersham).

Transcription assays

A derivative of the TAg Jurkat T cell line which expresses T-antigen was used for transcription experiments (TAg Jurkat T cells). TAg Jurkat T cells (1×10^7) were transfected with 1 μg of a reporter construct harboring SEAP under control of the NFAT enhancer. After transfection, these cells were resuspended in about 4 ml tissue culture media, and treated with 2 μg/ml PMA and 1 μg/ml ionomycin, to initiate production of SEAP. 100 μl aliquots of these cells were then exposed to different conditions, such as pure lipids, crude organic extracts, or compounds in various column fractions. After 24 h, SEAP activity was detected as described previously [2]. These assays were used to screen many column fractions quickly for the presence of apoptosis-inducing compounds.

Column chromatography

The organic extracts of two to three litters of mouse embryos were dissolved in a minimal volume of ethyl acetate. These extracts were loaded on a silica-gel column and eluted with 25% ethyl acetate/hexane. With a fraction size approximately one to two times the amount of silica gel used, the active compounds usually eluted between the fifth and fifteenth fractions collected. The fractions were dried under a nitrogen stream, resuspended in 20 μl ethanol, and tested for their ability to induce apoptosis in TAg Jurkat T cells.

The active compounds purified on silica gel were further purified on a recycling HPLC (JAI) using two tandem JAIGEL GS-310 columns. The active silica-gel purified fractions were pooled, dissolved in chloroform, and injected on the JAI HPLC. After one cycle on the JAI HPLC, many of the compounds in the mixture displayed overlapping elution profiles, with the active compounds having slightly faster retention times than average. The compounds with retention times similar to the active compounds were left to recycle on the JAI HPLC. After four cycles, several fractions of the recycled material were collected and their constituents analyzed by MS, IR and GC/MS, as described in the Results section.

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