Biosynthetic Regulation of Monobutyrin, an Adipocyte-secreted Lipid with Angiogenic Activity

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1-Butyrylglycerol (monobutyrin) is a novel angiogenic compound that is synthesized and secreted during the differentiation of 3T3-F442A preadipocytes into adipocytes. To study the regulation of monobutyrin biosynthesis we have developed an assay utilizing the glycerol kinase enzyme from Cellulomonas to quantitate the levels of this compound in cell-conditioned medium. Analysis of several cultured cell types, including tumor cell lines, indicated that monobutyrin production is detectable only from adipocytes, reaching a steady-state concentration of approximately 1.0 µM in conditioned medium. Monobutyrin synthesis was demonstrated in vitro using [14C]Butyryl-CoA with total homogenate or particulate fractions from adipocytes. Similar fractions from non-adipocyte cell lines failed to synthesize monobutyrin. This biosynthetic activity was shown to be distinct by substrate competition studies from the microsomal sn-glycerol-3-phosphate acyltransferase, whose activity is known to increase during adipocyte differentiation. The production of monobutyrin was hormonally regulated, as the addition of epinephrine to adipocytes caused a 10-fold increase in the amount of monobutyrin secreted. These results indicate that monobutyrin synthesis is adipocyte specific, occurs through an apparently novel particulate enzyme system, and is regulated in a hormone-dependent manner. The implications of these results for adipose physiology and angiogenesis are discussed.

Adipocytes play a key role in systemic energy balance, serving as the major depot for energy storage. The ability to store and recall metabolic energy in a biochemically useful form depends upon an intimate relationship between the adipose cells and the vasculature. Hence, the formation and function of new blood vessels are central to the development and physiology of adipose tissue. Adipocyte differentiation and angiogenesis are tightly coordinated during embryogenesis, and neovascularization is also required for further development of adipose tissue. Activation of lipolysis also brings about local vascular changes, most notably the dilation of small blood vessels and increased blood flow. Our previous work has shown that 3T3-F442A preadipocytes undergoing differentiation secrete factors that are strongly angiogenic in vivo and stimulate motility and mitogenesis of vascular endothelial cells in vitro.

One of these angiogenic factors was identified as the novel lipid 1-butyrylglycerol (monobutyrin) (6). The purified synthetic compound has been shown to promote angiogenesis in the chick chorioallantoic membrane assay and motility of isolated endothelial cells in vitro. Metabolic labeling studies utilizing various radiochemical precursors such as [14C]acetate have indicated that this compound is synthesized in a differentiation-dependent manner when adipocytes are compared with preadipocytes. Monobutyrin levels were estimated to increase 200-fold during the differentiation process. These results suggested that monobutyrin is a key regulatory molecule in the development of adipose tissue vasculature.

Little is known about the cell and tissue distribution of monobutyrin biosynthesis. The use of radiochemical labeling to estimate the concentration of a particular product quantitatively is subject to many assumptions regarding pool size, rate of uptake, and rate of clearance, which vary for each cell type. Furthermore, there is nothing known regarding the pathway of biosynthesis of this factor in adipocytes. To address these questions we have developed a facile enzymatic assay to investigate the production of monobutyrin by a variety of cell types and have also examined the enzyme(s) involved in the biosynthesis of this lipid angiogenic factor in broken cell preparations. These studies reveal that monobutyrin is synthesized by a novel enzyme or enzyme system and that the production of this lipid is regulated by both chronic and acute mechanisms in adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Glycerol kinases were purchased from Sigma. Pure monobutyrin was distilled from practical grade monobutyrin as described previously (6). 1-Butyryl-[3H]glycerol ([3H]monobutyrin) with a specific activity of 4.2 × 10⁶ cpm/nmol was a kind gift of Dr. Henry Lu (California Biotechnology). [γ-32P]ATP, [14C]Butyryl-CoA, and [2-14C]glycerol were purchased from Du Pont-New England Nuclear. sn-[14C]Glycerol 3-phosphate was synthesized from [14C]glycerol as described (7). Channeled silica gel thin layer plates were purchased from VWR. Sep-Pak C18 cartridges were purchased from Waters Associates.

Cell Culture—3T3-F442A cells were grown as described (8). Other cell lines were grown in DME plus 10% fetal calf serum unless otherwise indicated.

Purification of Monobutyrin from Conditioned Medium—Sep-Pak C18 cartridges were washed with 5 ml of glass-distilled water, activated with 5 ml of 95% ethanol, and washed again with 5 ml of glass-distilled water. One to 10 ml of conditioned medium from cells was applied through the column, the elute collected and passed through the column again. The cartridge was washed with 2 ml of glass-distilled water.

The abbreviations used are: monobutyrin, 1-butyrylglycerol; glycerol-P, sn-glycerol-3-phosphate; phosphomonomobutyrin, 1-butyryl-3-phospho-glycerol; DME, Dulbecco’s modified Eagle’s medium.
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Monobutyrin-The 50% ethanol eluate from the Sep-Pak fractionation was dried down under nitrogen gas and air dried completely. The plates were allowed to dry and were exposed overnight to Kodak XAR film. Bands corresponding to phosphomonobutyrin were scraped and counted in scintillation fluid.

Preparation of Cell Extracts—Cells were washed three times in ice-cold phosphate-buffered saline and scrapped in phosphate-buffered saline into 50-ml Falcon tubes. Cells were pelleted and resuspended in 5 volumes of homogenization buffer (0.25 M sucrose, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) and homogenized on ice with 20–30 strokes of a tight fitting Dounce homogenizer. Lysis was monitored by trypan blue staining to detect free nuclei. This suspension was centrifuged for 15 min at 15,000 g at 4 °C. The resulting supernatant was used as the whole cell homogenate. The homogenate was centrifuged at 400,000 × g for 20 min at 4 °C to obtain the cytosolic (supernatant) and particulate (pellet) fractions. The pellet was resuspended in 0.5 volume of homogenization buffer with 5–10 strokes of Dounce homogenizer and spun at 15,000 × g for 15 min at 4 °C. The resulting supernatant was used as the particulate fraction and was stored at -80 °C.

Assay of Monobutyrin Synthesis in Cell Extracts—Samples were assayed in 75 mM Tris-HCl, pH 7.4, and 10 mM MgCl₂ in the presence of various concentrations of radioactive precursors at 37 °C in a final reaction volume of 40 μl at the times indicated. The reactions were terminated by the addition of 5 μl of perchloric acid and immediately spotted onto channeled silica plates and air dried for at least 30 min. Plates were developed in ethyl acetate/isooctane/glacial acetic acid (11:5:2) saturated with water. Plates were then air dried and sprayed with En'Hance (Du Pont-New England Nuclear) before exposure to Kodak XAR-5 film.

Chemical Analysis of Phosphomonobutyrin and Monobutyrin—1-Butyryl-3-phosphoglycerol (phosphomonobutyrin) was isolated from bands visualized after autoradiography by scraping the silica and eluting with methanol. The resulting solution was then spotted on thin layer plates and developed in three solvent systems in which phosphomonobutyrin co-migrated with glycerol-P, glycerol, monobutyrin, or butyrate, indicating that a reaction was dependent upon the addition of enzyme, magnesium, and at least 0.1 mM ATP, all of which increased enzyme concentrations were somewhat inhibitory. This reaction was dependent upon the addition of enzyme, magnesium, and at least 0.1 mM ATP, although 5 mM appeared to be optimal.

Direct assays of conditioned medium for monobutyrin were not possible because of the presence of large amounts of glycerol and/or inhibitors of the glycerol kinase. An initial purification step was necessary to separate monobutyrin from these compounds. Quantitative recovery (97.4 ± 0.4%, n = 4) of labeled monobutyrin from cell culture medium was obtained by fractionation over Sep-Pak C18 cartridges. The recovery from Sep-Pak was quantitative at all concentrations examined (0.1–10 μM).

Using cell culture medium containing known amounts of added purified monobutyrin, the assay is linear up to 1,000 pmol (the highest amount tested) in a 50-μl reaction volume (Fig. 2). Monobutyrin levels of 25 pmol or less had a wide range of deviation and therefore were considered below the level of detection for this assay. The enzyme alone incubation (Fig. 2A, DME only lane) generated a band that co-migrated with phosphomonobutyrin. Base hydrolysis and subsequent thin layer chromatography indicated that this band was in fact phosphomonobutyrin, suggesting that the glycerol kinase preparation itself contained small amounts of monobutyrin. This background was subtracted out when determining monobutyrin values.

RESULTS

An Enzymatic Assay for Monobutyrin—There is currently no method available for measuring absolute amounts of monobutyrin. To study monobutyrin biosynthesis by cells in culture and its presence in various biological fluids, a simple quantitative method for measuring this molecule was developed. Our rationale was to use the glycerol kinase enzyme (EC 2.7.1.30) to phosphorylate the 3-hydroxy of monobutyrin (12, 13) with [γ-32P]ATP of a known specific activity and isolate the resulting [32P]phosphomonobutyrin from the reaction. To facilitate the screening of the enzymes, [1H]monobutyrin was used as a substrate to examine several commercially available glycerol kinases. Only one enzyme, that isolated from Cellulomonas sp., was able to phosphorylate monobutyrin. As shown in Fig. 1, this phosphorylation, which causes a change in mobility of the tritiated compound on the thin layer system, was essentially quantitative for monobutyrin levels ranging from 100 to 1,000 pmol in the assay. The kinase activity was linear with added enzyme to 400 units/ml, after which increasing enzyme concentrations were somewhat inhibitory. This reaction was dependent upon the addition of enzyme, magnesium, and at least 0.1 mM ATP, although 5 mM appeared to be optimal.

Using the enzyme alone incubation (Fig. 2A, DME only lane) generated a band that co-migrated with phosphomonobutyrin. Base hydrolysis and subsequent thin layer chromatography indicated that this band was in fact phosphomonobutyrin, dragging that the glycerol kinase preparation itself contained small amounts of monobutyrin. This background was subtracted out when determining monobutyrin values.

Fig. 1. Quantitative phosphorylation of [1H]monobutyrin by glycerol kinase. Increasing amounts of [1H]monobutyrin (100–1,000 pmol, as indicated in each lane) were incubated with 10 units of glycerol kinase from Cellulomonas and run on silica gel thin layer plates as described under “Experimental Procedures.” Arrows indicate where authentic monobutyrin and phosphomonobutyrin migrate. The no enzyme lane is 1,000 pmol of [1H]monobutyrin added to the reaction mixture with no enzyme added. The bands seen co-migrating with phosphomonobutyrin in the no enzyme lane are contaminants. [1H]Glycerol from the [1H]monobutyrin preparation. The identity of phosphomonobutyrin was verified as described under “Experimental Procedures.”
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In Vitro Synthesis of Monobutyrin by Cell Extracts—To examine the mechanism of monobutyrin synthesis, broken cell preparations of adipocytes were examined for the ability to synthesize monobutyrin in vitro. Adipocyte particulate and cytosolic fractions were incubated in the presence of 40 μM butyryl-CoA and radiolabeled glycerol-P (75 μM) as described under “Experimental Procedures.” Monobutyrin synthetic activity was evident and appeared to be membrane bound as the particulate fraction of adipocytes had an apparent specific activity of 0.73 nmol/mg whereas the cytosolic fraction contained no detectable activity. Monobutyrin synthesis was linear with time (up to 1 h) and protein (up to 250 μg). Synthesis was optimal at a pH range between 6.5 and 7.5 and was completely dependent on added magnesium. Monobutyrin synthesis was completely dependent upon the addition of butyryl-CoA if glycerol-P was used as the labeled substrate. However, monobutyrin synthesis was not dependent on or stimulated by the addition of glycerol or glycerol-P if [14C] butyryl-CoA was used as a substrate, suggesting that an endogenous glycerol backbone donor is present in the particulate fraction.

Although monobutyrin was detected only in adipocyte-conditioned medium (see Fig. 3), it was possible that part of the enzymatic machinery for monobutyrin synthesis was present in non-adipose cells. 3T3-F442A preadipocytes and NIH-3T3 fibroblast particulate fractions were examined for monobutyrin biosynthetic activity. The activity is seen only in membranes from adipocytes and not in the other cell types tested. In addition, particulate fractions made from adipocytes isolated from rat adipose tissue also demonstrated monobutyrin synthetic activity (not shown). Mixing experiments did not indicate the presence of an inhibitor in non-adipocyte membranes or whole cell homogenates using either [14C] glycerol-P or [14C]butyryl-CoA as substrate.

Acylation of glycerol-P by butyryl-CoA could conceivably be carried out by the microsomal glycerol-P acyltransferase, whose activity is known to increase greatly in differentiating 3T3-L1 (19, 20) and 3T3-F442A (21) preadipocytes. Glycerol-P acyltransferase optimally uses chain lengths of C16-C18 and has been reported to utilize acyl-CoAs of chain length 10

Secretion of Monobutyrin by Different Cell Types—Several different cell lines were examined for the ability to produce monobutyrin. Of the lines examined, including several transformed and tumor cell lines (e.g. sarcoma 180 and fibrosarcoma), only adipocytes (AD CM) made detectable amounts of monobutyrin (Fig. 3). In addition to the 3T3-F442A line, adipocytes derived from 3T3-L1 cells also made detectable amounts of monobutyrin (not shown). Based upon a limit of detection of 25 pmol, we estimate that cell-conditioned medium of non-adipose cells must contain a concentration of less than 10^{-6} M. The steady-state concentration of monobutyrin in 3T3-F442A adipocyte-conditioned medium was calculated to be 1.3 (± 0.1) x 10^{-6} M (n = 4).

**Fig. 2. Titration of monobutyrin in the glycerol kinase-based assay.** A, autoradiogram of thin layer plate after glycerol kinase reactions. Purified monobutyrin was added to DME plus 10% fetal calf serum and fractionated over Sep-Pak C18. Assays of various amounts of the monobutyrin-bearing fraction (20 pmol-1.0 nmol, as shown in each lane) were performed as described under “Experimental Procedures.” DME only refers to DME plus 10% fetal bovine serum without added monobutyrin. B, quantitation of the glycerol-kinase-based assay. An experiment similar to the one shown in A was performed. The regions corresponding to phosphomonobutyrin were scraped and counted. The resulting cpm were plotted versus the amount of monobutyrin added to the assay. The R coefficient for this line was 0.9967. The specific activity of the ATP used was 19.7 cpm/pmol.

**Fig. 3. Monobutyrin is specific to adipocyte-conditioned medium.** Medium conditioned for 24-48 h by several different cell lines at confluence was analyzed for the presence of monobutyrin as described under “Experimental Procedures.” The numbers shown correspond to conditioned medium volumes (in μl) after correcting for fractionation on the Sep-Pak cartridge. DME only, DME plus 10% serum; AD CM, adipocyte-conditioned medium; PREAD CM, preadipocyte-conditioned medium; NIH3T3 CM, NIH 3T3 (36) fibroblast-conditioned medium; S180 CM, sarcoma 180 (37) cell-conditioned medium (36); BPV1 CM, fibrosarcoma (38) cell-conditioned medium; REF CM, rat embryo fibroblast (39) cell-conditioned medium; 50 pmol MB, DME plus 10% fetal bovine serum containing 50 pmol of monobutyrin.

2 Several other cell lines, in addition to those shown in Fig. 3, were examined for the presence of monobutyrin in their conditioned medium. These lines are: C3H10T1/2 mouse myoblast cell line, both the myoblast and myotube cell types (14); CH310T1/2 CLB fibroblast cell line (15); 3T3-L1 preadipocyte cell line (16); βTC3 pancreatic β-insulinoma cell line (17); HL60 promyelocytic leukemia cell line (18). All the above mentioned cell types and lines did not have detectable amounts of monobutyrin in their cell-conditioned medium.
performed. Incubation of the in vitro monobutyrin synthetic reaction with acyl-CoAs of lengths C2–C12 showed a decreasing ability to compete with [14C]butyryl-CoA as the chain length increased beyond C8 (Fig. 4). Longer chain lengths were not used in the assay because of their strong amphipathic properties (23). In addition, butyryl-CoA failed to inhibit the microsomal glycerol-P acyltransferase activity from adipocyte particulate fractions when palmitoyl-CoA was used as the substrate (not shown). These data suggest that the formation of monobutyrin is unlikely to involve glycerol-P acyltransferase and appears to utilize a specific and possibly novel enzyme system.

Monobutyrin Biosynthesis Is Hormonally Regulated—As shown above, a major chronic regulator of monobutyrin synthesis is cell differentiation. Because changes in vascular state are known to occur during the activation of lipolysis (2), we asked whether a known lipolytic agent could modulate monobutyrin production in fully differentiated cells. Adipocytes were serum deprived overnight, stimulated with 10⁻⁷ M epinephrine, and the conditioned medium was assayed for monobutyrin. As seen in Fig. 5, the unstimulated level of monobutyrin increased up to 4 h. The concentration then remained constant at approximately 1.0 μM up to 16 h (the longest time point examined). However, upon stimulation of the cells with epinephrine, the initial rate of monobutyrin secretion increased about 8-fold compared with the unstimulated state. After 8 h the steady-state monobutyrin level was maintained at 8 × 10⁻⁶ M for the remainder of the time period examined. Quantitation of glycerol release into the medium showed an approximate 10-fold increase by the epinephrine-stimulated cells over control in the first 4 h, indicating the activation of lipolysis.

As shown in Table I, the effect of epinephrine was dose dependent, showing an increase in stimulation of monobutyrin from 1 × 10⁻⁹ M to 1 × 10⁻⁶ M. Two analogs of cAMP (dibutyryl and 8-bromo) (26) and prostaglandin E₁ (24, 25), two classes of lipolytic agents, also stimulated the synthesis of monobutyrin. However, prostaglandin E₁ was less effective at elevating monobutyrin in conditioned medium; prostaglandin E₁ was also a weaker lipolytic agent than either analog of cAMP or epinephrine as assayed by glyceral release (not shown). Therefore, the synthesis of monobutyrin by adipocytes appears to be subject to hormonal regulation involving a cyclic AMP-linked lipolytic pathway.

**Discussion**

Monobutyrin is a novel lipid with angiogenic activity which we identified previously from adipocyte-conditioned medium (6). As such, the ability to study the regulation of this factor is greatly facilitated by a quantitative assay for monobutyrin in conditioned medium and other biological fluids. We describe here a facile assay for measuring monobutyrin levels in cell-conditioned medium. This procedure requires only a simple one-step purification from medium and subsequent phosphorylation of the compound via glycerol kinase. By using this assay we have demonstrated that monobutyrin is present in micromolar quantities at steady-state levels in adipocyte-conditioned medium and is not detectable in the cell-conditioned medium of the other cell lines examined. We estimate that if monobutyrin is secreted by these cells it must be present at a concentration of less than 10⁻⁹ M in conditioned medium.

Although the use of the glycerol kinase assay indicates that
the secretion of monobutyrin is differentiation dependent, it is conceivable that the synthesis of the factor was not. We have demonstrated that the whole cell homogenates and particulate fractions isolated from adipocytes are able to synthesize monobutyrin in vitro. This synthetic activity appears to be adipocyte specific and can utilize glycerol-P, butyryl-CoA, and to a lesser extent, glyceral (not shown) as substrates. Use of this assay allowed us to establish that the activity has a preference for short chain fatty acyl-CoAs (see Fig. 5). This specificity ruled out the possibility that the activity was caused by the previously characterized microsomal glycerol-P acyltransferase, whose activity is known to increase greater than 70-fold during 3T3 differentiation (20, 21). Our data are consistent with previous work that has shown that this acyltransferase assayed from adipocytes failed to utilize acyl-CoAs of C10 chain length or shorter (22).

The simplest enzymatic mechanism to explain the synthesis of monobutyrin is either the acylation of glycerol-P by butyryl-CoA and subsequent dephosphorylation to monobutyrin or the direct acylation of glyceral with butyryl-CoA to form monobutyrin. These mechanisms would be analogous to the pathway of diacylglycerol synthesis (27) and consistent with the fact that radiolabeled glycerol-P and glyceral can be incorporated into monobutyrin. However, we have found that [14C]butyryl-CoA can be used as a substrate by washed adipocyte particulate fractions without any other added substrates. Furthermore, adding glyceral or glycerol-P to these washed membrane fractions with [3H]butyryl-CoA in the assay failed to increase the amount of monobutyrin produced. These preliminary results suggest that there must be an endogenous glycerol backbone generated from a compound present in the washed particulate fractions. The origin of the glycerol moiety utilized in monobutyrin synthesis is currently under examination.

In light of its angiogenic activity, the cell type specificity of the monobutyrin molecule is somewhat surprising. Conditioned medium from several cell lines representative of actively metabolizing tissue that is vascularized (tumor, muscle, and embryonic) showed that none contained detectable amounts of monobutyrin. This suggests that the monobutyrin molecule may play a specialized role in adipocyte physiology. Although the vascularization of adipose tissue could certainly utilize mechanisms distinct from other developing cell types, it is also possible that monobutyrin is a pleiotropic effector having multiple functions related to blood vessel biology, some specific for adipose cells. In this regard, the rate of blood flow and state of vasodilation in adipose tissue are both modulated in various metabolic states (2, 28). It is believed that increased blood flow facilitates the removal of fatty acids from adipose tissue during the fasting, lipolytic state (29). Interestingly, we show here that monobutyrin is acutely regulated by lipolytic factors.

Because monobutyrin production increases sharply at the onset of lipolysis, monobutyrin could be involved in some aspect of vasodilation and/or vascular permeability. The notion of a single molecule having both angiogenic activity and other biological actions is now well established. Fibroblast growth factor, tumor necrosis factor, and prostaglandins (just to name a few) have angiogenesis as only one of several biological activities (30-33). Similarly, vascular endothelial growth factor elicits many different responses even within the blood vessel systems, having been isolated independently as both an angiogenic factor (34) and a vascular permeability factor (35). Since angiogenesis appears to be a rather permissive biological response to many pharmacological effectors, the cell type specificity and acute hormonal control over monobutyrin synthesis suggest that this molecule may have other additional biological and physiological effects on vascular cells or on other cell types. These are currently under investigation.

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