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The concentration of free glycerol in goat milk increases during feed restrictions

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Research Article

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Abstract

This Research Communication introduces a novel enzymatic-fluorometric analytical procedure for glycerol and glycerol 3-phosphate in milk. Milk from thirty-seven goats was analysed during 9 consecutive days during which a two-day feed restriction was introduced. Fractional milk triacylglyceride and free glycerol increased significantly while glycerol 3-phosphate reacted more moderately. The energy status of the mammary cell is discussed.

Glycerol and glycerol 3-phosphate (glycerol 3P) are metabolites found ubiquitously in the mammalian organism, partly as free metabolites and partly incorporated into larger organic substances. Glycerol 3P may arise in connection with cytoplasmic glycolysis of carbohydrate, in the pentose phosphate pathway or from phosphorylation by glycerol kinase of glycerol obtained by lipolysis of triacylglycerols taken up by the cell. The glycerol 3P shuttle is a mechanism working across the mitochondrial membrane, regenerating NAD^+ from NADH and forming reducing equivalents consisting of $FADH_2$ in the mitochondrial matrix. In the mammary gland, glycerol 3P is a precursor for phospholipids and triacylglycerides, meaning that considerable amounts of triacylglycerides (TAG: Milk fat) are synthesized by esterification between glycerol 3P and acyl-CoA.

Both glycerol and glycerol 3P exist in a free form in milk. The objective was to develop analytical methods discriminating between glycerol and glycerol 3P in milk and investigate if the energy status of the lactating animal would affect the milk content of these metabolites.

Materials and methods

Thirty-seven goats (French Alpine) housed at the INRAE Experimental Installation of UMR 0791 MoSAR were kept in a feeding experiment for 17 d. Day 5 (afternoon) to 7 (afternoon) in experiment the ad libitum total mixed ration was substituted by straw only (ad libitum), resulting in a serious decline in available energy content and intake. Morning milk was sampled during 9 consecutive days, i.e. day 4–12 of the experiment. The research was carried out in compliance with French National Regulations for the humane care and use of animals for research purposes under license to carry out animal experiments: A 78 615 1002

Milk TAG was analysed according to Larsen *et al.*, 2011. Free glycerol and glycerol 3-phosphate were determined in two steps by enzymatic-fluorometric procedures. Analytical principle: Free glycerol is converted *via* glycerol kinase (ATP:glycerol 3-phospho-transferase; EC 2.7.1.30) to glycerol 3-phosphate. Glycerol 3-phosphate is further metabolized into 3-dihydroxyacetone phosphate and H_2O_2 *via* L-a-glycerophosphate oxidase (*sn*-glycerol-3-phosphate:oxygen 2-oxidoreductase; EC 1.1.3.21). H_2O_2 subsequently oxidizes ADHP (10-acetyl-3,7-dihydroxyphenoxazine) to the fluophore resorufin. Developed resorufin is measured fluorometrically, by excitation by 544 nm light and emission at 590 nm (FLUOstar Galaxy, BMG Technologies GmbH, D-77656 Offenburg Germany). This can be summarized as:

1). Glycerol + ATP → Glycerol 3-phosphate + ADP
2). Glycerol 3-phosphate + O_2 → 3-dihydroxyacetone phosphate + H_2O_2
3). ADHP + H_2O_2 → resorufin

Steps 1 to 3 together detects the sum of glycerol and glycerol 3P, wheras steps 2 to 3 alone detects only glycerol 3P. Free glycerol is calculated as the difference, ie free glycerol = (glycerol + glycerol 3P) – glycerol 3P. Standard curves were based on chemically pure glycerol for both determinations (0–500 µM and 0–250 µM, respectively). High and low controls likewise (152 and 48 µM, and 76 and 24 µM, respectively). Validation of the assays (accuracy, linearity, intra- and inter-assay precision) were conducted on control material (including glycerol free goat milk) and original goat milk. Accuracy was estimated on 4 times 8 replicates of control samples (inter plate). Linearity combined with recovery (glycerol + glycerol 3P) was tested on 96 goat milk samples (intrinsic content, min – max: 21–130 µM) spiked with standard material

(0–500 µM). Linearity of glycerol 3P (only) was determined by dilution (4 + 1 water) of 24 samples with a relatively high intrinsic level of glycerol 3P. Twenty-four milk samples were tested for intra- and inter-plate precision (three replicates × 3 plates) for the sum of glycerol and glycerol 3P and for glycerol 3P only.

Results

Accuracy: 152 µM controls on average gave 158.8 µM (i.e. 4.5% above the ideal target; inter- CV% = 2.3). The low control on average gave 45.9 µM (i.e. 4.4% below target; inter- CV% = 7.0). Correspondingly, for the glycerol 3P analyses: high control average 79.9 µM (plus 5.2% compared to target; inter- CV% = 4.3); low control 23.4 µM (2.5% lower than target; inter- CV% = 5.0).

For linearity analysis of (glycerol + glycerol 3P; spiking): the measured mean value of 96 standard additions was 127.5 µM (min–max: 26.0–324.3) whereas the calculated (expected) value was 122.1 µM (min–max 21.8–289.1). The regression equation was: measured value = 1.07 × exp. value − 3.5 µM; $R^2 = 0.985$, $n = 96$). For linearity analysis of (glycerol 3P; dilution with water): the diluted samples (range 23.6–86.1 µM) contained on average 79% of the initial value (min–max: 69–86%). Regression against the original samples: diluted result = 0.81 × undiluted result − 1.2 µM; $R^2 = 0.982$; $n = 24$). The combined glycerol + glycerol 3P assay showed an intra-assay CV% precision of 2.9; $N = 72$; $n = 3$). Inter-plate CV% was 4.5 (min–max: 0.6–13.1; $N = 72$, $n = 3$). Correspondingly for the glycerol 3P assay: intra-assay CV%: 3.8 ($N = 72$, $n = 3$); inter-plate assay CV%: 7.1 (min–max: 1.8–11.7). The lowest standards for both assays (20 resp. 10 µM) were highly significant from the 0-standard, indicating that the detection limit of the method is at least this level. None of the samples analysed in this presentation were below this limit.

The TAG concentrations in milk are shown in Fig. 1. They increased from an average of 64 mM day 6–112 mM day 7 ($P < 0.001$; $LSD_{0.99} = 9.0$), i.e. an instant increase (75%) in fractional TAG content approximately 38 h after feed change. The concentration returned quickly to the point of origin only 16 h after refeeding with TMR rations. Free glycerol concentration increased rapidly from day 6 to 7, i.e. 16 h after feed change to 40 h after (70%), and further from 57.7 to 91.5 µM during the next 24 h (60%, $P < 0.001$; $LSD_{0.99} = 22.7$) and declined steadily hereafter. By day 10 of the experiment the glycerol content was not significantly different from the level at day 4 and 5. Milk glycerol 3P increased from day 6 to 7 (36.0–51.4 µM; $P < 0.001$, $LSD_{0.99} = 12.1$), but from a more variable basic level (days 5, 7, 8, and 9 were not significantly different in glycerol 3P concentration: Fig. 1).

Discussion

Several analytical procedures for glycerol (especially) and glycerol 3P have been introduced (ultraviolet methods, luminometric, radiometric: see Bergmeyer, 1988), but these methods are not appropriate for an opaque matrix like milk. Methods based on advanced analytical equipment like capillary gas chromatography, high performance liquid chromatography (optionally coupled to isotope ratio mass spectrometry, see Jung *et al.*, 2006) require a thorough pretreatment of the sample, which is not necessary in the present enzymatic-fluorometric method, and which also operates in opaque matrices (Larsen and Moyes, 2010).

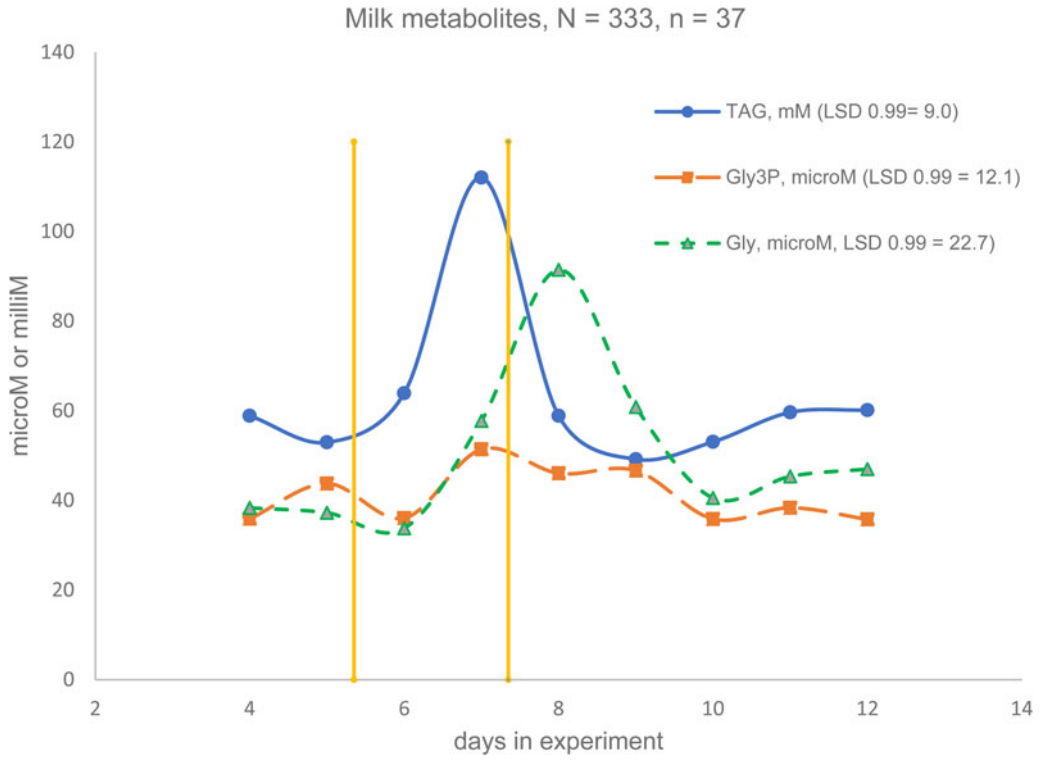

Figure 1. Thirty-seven goats were kept in a feeding experiment for 17 d. Day 5 (afternoon) to 7 (afternoon) in experiment the *ad libitum* total mixed ration was substituted by straw only (*ad libitum*), resulting in a serious decline in available energy content and intake. Morning milk was sampled during 9 consecutive days, i.e. days 4–12 in trial. Milk triacylglyceride (TAG), free glycerol, and glycerol 3-phosphate were analysed by enzymatic–fluorometric procedures.

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In normally fed goats it is generally accepted that approximately 50% of milk fatty acids are absorbed from the blood and another 50% are *de novo* synthesized in the mammary cytosol. Adhesion of minor chylomicrons to the walls of the luminal surface of blood capillaries seems to be facilitated by lipoprotein lipase, and hydrolysis by this enzyme is a prerequisite for uptake by the mammary cells. Arterio-venous measurements have shown negligible difference in free fatty acids and only minor net absorption of glycerol across the mammary tissue. However, arterio-venous studies using labelled metabolites have demonstrated a release of free fatty acids and free glycerol by mammary cells (Annison *et al*., 1968; West *et al*., 1972). In the starved animal, on the contrary, the net absorption of free fatty acids increases and the absorption of triglycerides decreases considerably, resulting in an uptake/output ratio <1 of fatty acid carbon over the mammary tissue (reviewed by Annison, 1983). In addition, starvation decreases the rate of *de novo* fatty acid synthesis in the mammary gland, resulting in the production of milk fat consisting of predominantly long-chain fatty acids (Annison *et al*., 1968; Pires *et al*., 2022) presumably with a huge contribution from free fatty acid uptake. A relative increase in milk fat concentration and a reduced milk production are well documented during reduced energy intake (Friggens *et al*., 2016). Feed restriction periods, however, lead to only nominally reduced milk fat output (weight, fatty acid carbon and glycerol). Even though the milk fat production declines during feed restrictions, more carbon building blocks are apparently taken from the milk fat precursors in the mammary tissue itself (reviewed by Annison, 1983).

It is reasonable to believe that TAG and the intermediate metabolites in milk are reflecting the energy situation in the mammary cell at the time of secretion. Under fed conditions, acetate, lactate, pyruvate, betahydroxy butyrate (BHB) and, to a limited extent, glucose are known to contribute to *de novo* FA synthesis (Forsberg *et al*., 1985a; Drackley, 2000). The fractional use of these carbon-building blocks seems to be dependent upon the relative concentration of the metabolites (Forsberg *et al*., 1985a, 1985b) and regulation of fatty acid synthase is largely through intracellular concentrations of dietary or synthesized fatty acids, which decrease its activity (Smith, 1994). In energy deficiency, mainly BHB and intermediates from the glycolysis and pentose phosphate pathway would appear in increased quantities. The fractional increase of free glycerol and (to a certain extent) glycerol 3P suggests either a spontaneous surplus of these metabolites during reduced available energy, or a constant liberation during reduced milk volume. The incorporation of glycerol 3P into TAG is reduced, pointing towards an accumulation of glycerol. Another mechanism could be the acceleration of gluconeogenesis from lactate, amino acids and other metabolites, creating a deficiency of carbon-building blocks for fatty acids to be incorporated in TAG, although this mechanism would be energetically expensive in an energy deficient cell.

In conclusion, a novel enzymatic–fluorometric analytical procedure for glycerol and glycerol 3-phosphate in milk has been developed. During restricted feeding, fractional milk triacylglyceride and free glycerol increased significantly while glycerol 3-phosphate reacted more moderately. This has enabled speculation regarding the energy status of the mammary cell during whole-body energy defecit.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029924000396

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