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Effects of putrescine, cadaverine, spermine, spermidine and β-phenylethylamine on cultured bovine mammary epithelial cells

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ABSTRACT

A bovine mammary epithelial cell line (BME-UV1) and three-dimensional collagen primary bovine organoids were used to evaluate the effects of cadaverine, putrescine, spermine, spermidine and β-phenylethylamine on mammary epithelial cells. Each biogenic amine was diluted in several concentrations (0-50 mM in BME-UV1 and 0-4 mM in primary bovine organoids) in the appropriate saline solution for the cell culture considered. In order to determine the activity of each compound tritiated thymidine incorporation was used. At low concentrations, all amines induced cell proliferation in both cultures. In BME-UV1, spermine significantly inhibited cell proliferation (P<0.001), while the other amines inhibited at higher concentrations (50mM). In primary bovine organoids, β−phenylethylamine significantly (P<0.001) inhibited cell proliferation at 4 mM. Organoids cultured in the presence of all amines, except β-phenylethylamine, had stellate projections indicating intense cell proliferation.

Proliferation of mammary epithelial cells was stimulated at low concentrations, while at high concentrations it was inhibited. Our results suggested that the effects of each compound on mammary epithelial cells could be related to the compound itself and not to mediating by the bovine amino oxidase, responsible of the formation of toxic metabolites.

Key words: Biogenic amines, Cell cultures, Bovine mammary gland.

RIASSUNTO

EFFETTI DI CADADERINA, PUTRESCINA, SPERMINA, SPERMIDINA E β-FENILETILAMINA SU COLTURE CELLULARI DI GHIANDOLA MAMMARIA BOVINA

Al fine di valutare l’effetto esercitato da cadaverina, putrescina, spermina, spermidina e β-feniletilamina sulle cellule di ghiandola mammaria bovina, sono stati impiegati due modelli in vitro, la linea cellulare...
BME-UV1, costituita da cellule epiteliali di ghiandola mammaria bovina, e colture primarie di organoidi bovini mantenuti in collagene.
Ciascuna amina biogeno è stata risospesa in diverse concentrazioni (0-50 mM nelle BME-UV1 e 0-4 mM negli organoidi primari bovini) nella salina base propria per la coltura cellulare considerata. Per determinare l’attività sostenuta da ciascun composto è stata impiegata l’incorporazione di timidina triziata. A basse concentrazioni, tutte le amine biogene sono state in grado di indurre la proliferazione cellulare in entrambi i sistemi in vitro considerati. Nelle cellule BME-UV1, la spermina inibisce significativamente la proliferazione cellulare ($P<0,001$), mentre tutte le altre amine sono in grado di indurre un’inibizione della proliferazione cellulare solo ad alte concentrazioni (50mM). Nelle colture primarie di organoidi, invece, è la β-feniletilamina in concentrazione pari a 4 mM ad inibire significativamente ($P<0,001$) la proliferazione cellulare. Gli organoidi primari bovini mantenuti in presenza delle diverse amine biogene, tranne per la β-feniletilamina, hanno presentato proiezioni stellate, indice di una intensa proliferazione. Dal nostro lavoro emerge come nei modelli considerati la proliferazione delle cellule epiteliali è stata stimolata a basse concentrazioni, mentre ad alte è stata inibita. Considerando che tali risultati sono emersi in esperimenti condotti in assenza di siero bovino, l’attività mostrata da ciascun composto è imputabile al composto stesso e non alla mediazione sostenuta dall’amino ossidasi bovina, responsabile della formazione di metaboliti tossici.

Parole chiave: Amine biogene, Colture cellulari, Ghiandola mammaria bovina.

Introduction

Amines are formed during normal metabolic processes in all living organisms and are present in feed and food. Dietary amines can be classified on the basis of their synthesis as natural polyamines (putrescine, spermine, spermidine, cadaverine) that are formed during de novo polyamines biosynthesis, and biogenic amines (β-phenylethylamine, putrescine, cadaverine) formed by non-specific decarboxylation reactions (Bardóczi, 1995). In feedstuffs biogenic amines are mainly produced by the degradation of plant proteins and amino acids during storage (Silla Santos, 1996). Putrescine, cadaverine, spermine, spermidine and β-phenylethylamine are the most widespread biogenic amines. Their presence in high concentrations limits the nutritional value of feedstuffs and their excessive consumption may have adverse effects on animal (Fusi et al., 2004) and human health (Bardóczi, 1995). For example, β-phenylethylamine raises blood pressure (Stratton et al., 1991), and spermine and spermidine can form carcinogenic nitrosamines by reaction with nitrite (Halász et al., 1994).

However, it is difficult to establish toxicity thresholds for these substances as the toxicity of the individual amines varies, and also depends on their interactions with other compounds (Silla Santos, 1996). Furthermore, the polyamines putrescine, spermine and spermidine are present in all cells at low levels, and play fundamental roles in DNA, RNA and protein synthesis ( Löser, 2000).

Motyl et al. (1995) and Ploszaj et al. (1997) investigated polyamine levels in milk from goats, sows and cows. Levels peak in the colostral phase and early lactation. Similarly, in human milk spermidine and spermine concentrations rise markedly during the first 3 days of lactation, to plateau at around 10 times higher than day 0 levels (Buts, 1998).

These findings not only suggest a relation between polyamine secretion and the intense mammary gland activity during the lactation, they are also indicative of the demand of newborn animals for these polyamines that peaks at this time (Motyl et al., 1995). In fact considerable evidence indicates a role of polyamines in gut development and regulation of metabolism in newborn ani-
Ital. J. Anim. Sci. vol. 7, 131-140, 2008

Biogenic amines in bovine mammary cells

Biogenic amines in bovine mammary cells (Gréco et al., 2001). Most mammalian cells can synthesise polyamines, but biosynthetic capacity may not always meet requirements (Pegg, 1986). Diet and the bacterial intestinal production may be major sources of the polyamines present in milk (Bardóczy et al., 1993; Motyl et al., 1995).

Though the importance of polyamines for growth and development of newborns is studied, and their presence in milk well documented, there is no information on the effects of these compounds and other amines on the growth and development of mammary epithelial cells.

The studies on the role sustained by some biogenic amines are many, including several approaches to the argument. Such effects can be investigated in mammary cell cultures as they provide a homogeneous in vitro model that directly focuses on the epithelial cell. Recently the use of in vitro models has gained more attention in the scientific community, in relation to the possibility to reduce the use of animals for research (3R, as there are Reduction, Refinement and Replacement of animal experiments). This could involve the animal production area, where the animal condition and welfare are some of the main points to be considered. Mammary cell cultures have been successfully used to evaluate effects of mycotoxins (Baldi et al., 2004) and biogenic amines (Cheli et al., 2001). However, cell lines do not have the biological variability and metabolic complexity of freshly isolated cells (Matitashvili et al., 1997), and the use of primary bovine organoids – a three-dimensional model of the mammary gland – may better approximate the complexity and variability of the mammary gland in vivo (Purup et al., 2000a, 2000b).

The aim of the present study was to evaluate the effects of some biogenic amines on mammary epithelial cell proliferation and development using different in vitro models, such as a bovine mammary epithelial cell line (BME-UV1) and primary bovine mammary epithelial cells (organoids).

Material and methods

BME-UV1 cells were cultured in plastic Petri dishes at 37 °C in an incubator, in a humidified atmosphere containing 5% CO₂ (Cheli et al., 2003). The culture medium was a 5:3:2 mixture of DMEM/F12, RPMI-1640, and NCTC-135 supplemented with 10% FBS, 0.1% lactose, 0.1% lactalbumin hydrolysate, 10 µg/ml ascorbic acid, 1.2 mM glutathione, 1 µg/ml hydrocortisone, and 1 µg/ml insulin (Sigma, St. Louis, MO, USA). Cell viability was preliminarily detected by trypan blue exclusion. To assay BME-UV1 proliferation, cells were seeded at 4x10⁴ cells/well into 96 well-plates in culture medium. After 24 hours the cells were washed and incubated with treatment medium, DMEM/F12 containing different concentrations (0-50 mM) of putrescine, cadaverine, spermine, spermidine or β-phenylethylamine. After 72 hours of treatment, 2.5 µCi/well of [methyl-3H] thymidine (0.0925 MBq/250 µl; Amersham Biosciences, Hørsholm, Denmark) was added to each well. Cell proliferation was determined six hours later by measuring tritiated thymidine incorporation (Purup et al., 2001).

To prepare organoids, aseptically excised pieces of mammary parenchyma from pre-pubertal (8-9 month-old) Friesian heifers were digested in basal medium (M199 with Earles salts; Sigma, St. Louis, MO, USA) supplemented with type II collagenase (Worthington Biochemical Corp., Freehold, NJ, USA), hyaluronidase, DNase and bovine insulin (all Sigma) (Purup et al., 2001). Basal medium was a serum-free M199 containing BSA (2.6 g/l), transferrin (5 mg/l), reduced glutathione (1 mg/l), soybean trypsin inhibitor (1 mg/l), bovine insulin (10 µg/l), selenium (1 µg/l) and antibiotic solution...
(0.2%) containing penicillin (50000 IU/l), streptomycin (50 mg/l) and amphotericin (125 µg/l).

Organoids were isolated by filtration, centrifugation and precipitation; preparations from each heifer were collected and frozen in liquid nitrogen pending use.

Organoids were quickly thawed at 37 °C washed in basal medium to remove DMSO, centrifuged and resuspended in cold neutralised rat collagen solution (1:1 v/v in basal medium) prepared from rat tails as described by Purup et al. (2001). A 1:1 mixture of cell suspensions from two heifers was then prepared. Previous studies (Purup et al., 2000a) have shown that organoids produced from different heifers of about the same live weight respond very similarly to growth factors, serum and mammary tissue extracts.

The cellular collagen suspension (0.5 ml) of mixed cell suspension were pipetted into the wells of 24-well plates (Nunc A/S, Roskilde, Denmark) previously coated with 0.5 ml of neutralised collagen solution. The suspension was allowed to gel in a 37 °C incubator for 1 h following which 1 ml basal medium was added.

Organoids were cultured for 3 days in 24 well plates at 37 °C in 5% CO₂.

On day 4, basal medium was discharged and treatment medium was added and cells were incubated for 24 hours. Treatment medium consisted of a saline solution (M199) containing putrescine, cadaverine, spermine, spermidine or β-phenylethylamine (all from Sigma) in the range 0.0009 mM to 4 mM. The solutions were prepared by serial dilution of amine stock solutions (4 mM). On day 5, 1 µCi/well of tritiated thymidine (0.037 MBq/ml Amersham) was added to each well for a further 24 h and cell proliferation was determined as for BME-UV1 cells (Purup et al., 2001). In order to observe changes in colony shape during the experiment, culture wells of organoids from each treatment group were examined at the end of thymidine incorporation. A Leica microscope mounted with a Sony video camera was used for time-lapse light examination of individual colonies of organoids.

Three replicates at each amine concentration in each culture type were obtained in each experiment. All experiments were repeated at least twice. Because of variation in the mitogenic response to culture medium in independent assays, the results were adjusted by dividing values for thymidine incorporation per well by the average culture value (control) for incorporation in each assay. Data were expressed as least square means and standard errors. The results were analysed using the GML procedure of SAS version V8 (SAS Institute Inc., 1999).

The effects of treatments were assessed by one way ANOVA; differences with P<0.001 were considered significant.

Results and discussion

The use of different cellular models, based on the same constituent element, the bovine mammary epithelial cell, may offer an interesting approach to the study of a productive organ such as bovine mammary gland. Two bovine in vitro models were selected.

The use of BME-UV1 cell line provided advantages in relation to the easy and economic culture management, as well as the useful information on toxicity, although this in vitro model did not mimic the in vivo situation as a three-dimensional model does. A three-dimensional model of primary bovine organoids offers a very sensitive bioassay based on primary culture of undifferentiated bovine mammary cells in serum-free medium (Purup et al., 2000a). Using this bioassay, the mitogenic effects of a large number of different hormones and growth factors have been tested. The bioassay also is able
to detect changes in the effect of serum and mammary extracts caused by in vivo treatments such as different feeding levels (Sejrsen et al., 2000; Purup et al., 2000b), stage of development (Norup et al., 1997), exogenous growth hormone (Sejrsen et al., 1999), and changes in milk from different lactation stages (Sejrsen et al., 2001). Therefore, this bioassay is a very sensitive model for studying effects of different compounds, but also a good model of the mammary gland.

Organoids embedded in collagen gel represent a three-dimensional culture, a dynamic in vitro model that mimic cell-cell interactions, growth physiology and metabolic pathways in vivo, enhancing the study of the amines effects in a target organ as the mammary gland.

Our results indicated that the selected biogenic amines modulated cell growth in both mammary gland models considered. The effects of increasing concentrations of biogenic amines on tritiated thymidine incorporation of BME-UV1 cells are shown in Table 1. Data are expressed as mean incorporation value compared to control (no amine) after 72 hours of incubation with amine (cell proliferation). In BME-UV1 cells we used amine concentrations in the range 0.4-50 mM in order to demonstrate effects. From the Table 1, it is evident that thymidine incorporation (and hence cell proliferation) was affected in a dose-dependent manner by all added amines. In most cases there was a proliferative effect at low concentrations, while dose-dependent inhibition at higher concentrations was observed.

For all amines except spermine, thymidine incorporation was increased compared to control at low amine concentrations, but was lower than control at higher concentrations. In particular, putrescine and cadaverine had a proliferative effect up to 10 mM. Beta-phenylethylamine had proliferative effect up to 2 mM, but was cytotoxic thereafter. Spermine inhibited cell growth at all concentrations and did so in a dose-dependent manner. Thymidine incorporation data in cultured organoids after incubation with amine for 48 hours are presented in Figure 1. We found that in organoids putrescine, cadaverine and spermidine had no dose-dependent proliferative effects, that spermine had relatively dose independent proliferative effects except at the highest concentration, and that β-phenylethylamine had mainly a dose-dependent anti-proliferative effect.

Putrescine, in fact, had a small positive effect on cell growth at most concentrations,

| Table 1. Effects of amines on BME-UV1 cells after 72 h of incubation. |
|-----------------|-------|-------|-------|-------|-------|
|                 | CTR   | 0.4 mM| 2 mM  | 10 mM | 50 mM |
| Putrescine      | 1 ± 0.03^A | 1.04 ± 0.03^A | 1.12 ± 0.03^A | 1.28 ± 0.03^B | 0.07 ± 0.03^C |
| Cadaverine      | 1 ± 0.03^A | 1.31 ± 0.03^A | 1.31 ± 0.03^A | 1.17 ± 0.03^D | 0.04 ± 0.03^E |
| Spermidine      | 1 ± 0.03^A | 1.13 ± 0.04^A | 1.30 ± 0.04^B | 0.15 ± 0.04^C | 0.01 ± 0.03^D |
| β-phenyl^1      | 1 ± 0.03^A | 1.08 ± 0.04^A | 1.47 ± 0.03^B | 0.20 ± 0.03^C | 0.08 ± 0.03^D |
| Spermine        | 1 ± 0.03^A | 0.80 ± 0.03^B | 0.05 ± 0.03^C | 0.01 ± 0.03^D | 0.01 ± 0.04^E |

^1β-phenylethylamine

Data are expressed as least square means ± standard errors, obtained from cultures with triplicate samples. Values are presented as relative to proliferation obtained in culture medium (control). Within a row, least square means not sharing the same superscript letter differ significantly (P<0.001), compared to the control.
but it was not significant in the considered concentrations. The diamine cadaverine also increased cell growth at all concentrations generally by about 15%, without any significant effects. Spermine significantly \((P<0.001)\) stimulated the cell proliferation by about 30% at low concentrations \((0.0009-0.0070 \text{ mM})\). At concentrations in the range \(0.0018-0.0070 \text{ mM}\) it had little effect, but was cytotoxic at 4 mM when it reduced cell proliferation by 24% \((P<0.001)\) compared to control. Spermidine positively \((P<0.001)\) affected the growth of organoids, increasing proliferation by 35% compared with the con-
control cells. Beta–phenylethylamine had different effect depending on concentration. At low concentrations (0.0018-0.0150 mM) cell growth was similar to that in control, but at higher concentrations there was a cytotoxic effect (P<0.001) with reduced proliferation by 92% at 4 mM.

In organoids the amines produced different effects at considered concentrations. We carried out systematic experiments using concentrations in the range 0.0009-4 mM, which is a similar range to that of the concentrations of amines in colostrum and milk, as described by Motyl et al. (1995). Those Authors speculated that the presence of spermine and spermidine in milk was in relation to the requirement of the newborn. Spermine and spermidine support cell growth and proliferation, playing a fundamental role in the development of gastrointestinal tract and gut maturation (Löser, 2000).

Morphological observations of organoids revealed colonies with stellate projections, indicating intense cell proliferation, in the presence of putrescine (Figure 2A), cadaverine (Figure 2B), spermine (Figure 2C) and spermidine (Figure 2D). In the presence of high concentrations of β-phenylethylamine most organoids presented only few branches among them (Figure 2E), particularly evident in comparison to organoids cultured in basal medium (Figure 2F), which also had a broadly stellate appearance characteristic of the proliferating state. Although the effect on cell proliferation was not significant, the morphological aspect of organoids, induced by amine treatments corresponded to the presence of epithelial outgrowths in most of the colonies considered. The branches emerged from organoid surfaces and constituted a network of connections among the colonies.

Our results on the effects of amines on bovine mammary cell proliferation were consistent with the results obtained by other Authors in other in vitro models. Wallace et al. (1981) observed that at the maximum concentration used (2.5 mM), spermine was able to determine 58% inhibition of DNA synthesis in baby hamster kidney cells. Spermidine caused a small increase in nuclear DNA synthesis at low concentration (0.5 mM) and inhibition at higher concentration (2.5 mM). Moreover, Kleczkowska et al. (1981), using an in vitro pregnant rabbit mammary gland model, showed an inhibitory effect when spermidine (10^{-4}M and 10^{-3}M) and spermine (10^{-4}M and 10^{-3}M), respectively, were added. As mentioned above, the in vitro cytotoxicity of spermine and spermidine is documented in other cell lines. In the presence of serum amine oxidase which is found in bovine serum, Sharmin et al. (2001) reported on intracellular polyamine accumulation and inhibition of cell growth.

Using established cell lines He et al. (1993) demonstrated that 2 mM spermine and 10 mM spermidine inhibited more than 90% of cell growth after an incubation of 120 hours. Moreover, high concentration of putrescine (data not shown by the Authors), which was not a substrate for amine oxidase in serum, inhibited cell growth. Our results indicated that polyamines directly inhibited cell proliferation and induced cytotoxicity without the presence of amine oxidase because all the experiments were conducted in absence of serum. So it was hypothesized that the considered amines themselves could exert the toxic effects on cells, not their oxidative metabolites deriving from the activity of ruminant serum amine oxidase.

In a review, Wallace et al. (2003) discussed the role of polyamines as intracellular growth factors, able to increase the rate of cell growth and differentiation and support cell growth or death in relation to the cellular environmental signal. In the case of high
Figure 2. Morphology of organoids cultured in basal medium (control) and in the presence of biogenic amines diluted in saline solution (M199).

Putrescine (0.0037 mM) (A) and cadaverine (0.0037 mM) (B) stimulated the organoid growth, they developed stellate colonies, in communication with each other. Spermine (0.0070 mM) (C) and spermidine (0.0018 mM) (D) at low concentrations favoured the development of growth and connections between organoids. Beta-phenylethylamine was the only amine able to describe a dose response curve in the range of considered concentrations. At the highest concentration used (4 mM) (E) the presence of little smooth organoids was evident in most of the observed wells. This effect was more evident when compared to control wells (F) where epithelial outgrowth emerged from organoid surfaces. Cellular magnification x40.
exogenous polyamines concentrations, cells could lead to death through apoptosis process (Brunton et al., 1991).

Conclusions

We investigated the effects of biogenic amines on two types of bovine epithelial cell cultures to throw light on the effect of these amines on mammary gland.

Most of the previous studies conducted in vivo have focused on milk composition in pol- yamines in relation to their role in the gut development and maturation of the newborns (Motyl et al., 1995; Ploszaj et al., 1997). The in vitro studies in mammary glands in mice (Kano and Oka, 1976; Oka and Perry, 1983) indeed investigated the transport and the metabolism of these compounds. We found that the amines tested had both proliferative and cytotoxic effects. In conclusion, our results taken together with results of other Authors indicate that amine effects were cell type specific and related to cellular concentration, leading proliferative or cyto- toxic effects, both in a developed and mature epithelia (BME-UV1) and in primary organoids derived from prepuberal heifers.

Abbreviations:
BME-UV1, bovine mammary epithelial cell line; M199, modified HEPES with Earle’s salts; BSA, bovine serum albumin; DMEM/F12, Dulbecco’s modified Eagle’s medium/F-12 HAM nutrient mixture; RPMI-1640, RPMI-1640 culture medium; NCTC-135, NCTC-135 medium; FBS, foetal bovine serum.

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