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Research paper

Assessment of Poly(ADP-ribose) Polymerase1 (PARP1) expression and activity in cells purified from blood and milk of dairy cattle

Giovanna De Matteis[a,*,1], Anna Reale[b,1], Francesco Grandoni[a], Mirella L. Meyer-Ficca[c], Maria Carmela Scatà[a], Ralph G. Meyer[c], Luca Buttazzoni[a,2], Bianca Moioli[a,2]

[a] Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA)– Centro di ricerca Zootecnia e Acquacoltura, Via Salaria, 31– Monterotondo, Rome, Italy
[b] Dipartimento di Biotecnologie Cellulari ed Ematologia, Sezione di Biochimica Clinica, Università "La Sapienza", Rome, Italy
[c] Department of Animal, Dairy and Veterinary Sciences, College of Agriculture and Applied Sciences, Utah Agricultural Experiment Station, Utah State University, Logan, UT, USA

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ABSTRACT

Poly(ADP-ribosyl)ation (PAR) is a post-translational protein modification catalysed by enzyme member of the poly(ADP-ribose) polymerases (PARPs) family. The activation of several PARPs is triggered by DNA strand breakage and the main PARP enzyme involved in this process is PARP1. Besides its involvement in DNA repair, PARP1 is involved in several cellular processes including transcription, epigenetics, chromatin re-modelling as well as in the maintenance of genomic stability. Moreover, several studies in human and animal models showed PARP1 activation in various inflammatory disorders. The aims of the study were (1) to characterize PARP1 expression in bovine peripheral blood mononuclear cells (PBMC) and (2) to evaluate PAR levels as a potential inflammatory marker in cells isolated from blood and milk samples following different types of infection, including mastitis. Our results show that (i) bovine PBMC express PARP1; (ii) lymphocytes exhibit higher expression of PARP1 than monocytes; (iii) PARP1 and PAR levels were higher in circulating PBMCs of infected cows; (iv) PAR levels were higher in cells isolated from milk with higher Somatic Cell Counts (SCC > 100,000 cells/mL) than in cells from milk with low SCCs. In conclusion, these findings suggest that PARP1 is activated during mastitis, which may prove to be a useful biomarker of mastitis.

1. Introduction

Poly(ADP-ribosyl)ation is a post-translational modification of cellular proteins highly induced after DNA damage. The reaction is catalysed by poly(ADP-ribose) polymerases (PARPs) (EC 2.4.2.30), an enzyme family using NAD^+ as substrate to form polymers of ADP-ribose (PAR). Poly(ADP-ribose) polymerase1 (PARP1) is the best studied family member which is responsible for approximately 90% of cellular PAR formation after genotoxic stress (Bürkle et al., 2004). PARP1 is a 116 kDa nuclear protein that is well conserved during evolution (Uchida and Miwa, 1994) with a 95% overall amino acid sequence similarity among mammalian species (Beneke et al., 1997). Bovine PARP1 shares 98% similarity with the human enzyme and the major caspase cleavage sites are conserved (Saito et al., 1980). Besides DNA repair, transient PAR formation has also been involved in several other cellular processes including control of epigenetic profile (Reale et al., 2005), transcription, chromatin re-modelling (Posavec Marjanovic et al., 2017; Sultanov et al., 2017), maintenance of genomic stability (Bai, 2015) and apoptosis (Valenzuela et al. 2002). In cases of inflammation resulting in extensive tissue damage, PARP1-mediated PAR synthesis may be excessive and lead to the cellular depletion of its substrate NAD^+ (Berger, 1985) as well as to cell death signaling (D'Amours et al., 2001). Several types of cellular stress, including genotoxic (DNA damaging) agents such as peroxynitrite formed in inflammatory processes from nitric oxide (NO), trigger a programmed cell death pathway, called apoptosis (Brüne, 2005). Apoptosis involves the activation of caspases to cleave proteins during programmed cell death. PARP1 cleavage by Caspase-3 has been regarded as a reliable marker of apoptosis in cellular research for many years (Lazebnik et al., 1994). In this study we have focused on changes in PARP1 activity associated with inflammatory responses of the bovine udder.

Mastitis, the inflammation of mammary gland, is a serious disease that causes huge economic losses to the dairy industry (Rollin et al., 2015) and has become one of the main research topic in the field of

* Corresponding author.
E-mail address: giovanna.dematteis@crea.gov.it (G. De Matteis).
[1] Both authors contributed equally to the manuscript.
[2] Both authors contributed equally to the manuscript.

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dairy science due to the financial losses generated and food safety concerns over antimicrobial use. Mastitis usually occurs as an immune response to bacterial invasion of the teat canal by a variety of bacterial sources present on farms. Following pathogen invasion, the resident leukocytes together with epithelial cells initiate the inflammatory response required to eliminate the invading bacteria (Paape et al., 2003; Rainard and Riollet, 2006). These cells release chemoattractants for the rapid recruitment of polymorphonuclear leukocytes (PMN) to the site of infection. The PMN eliminate pathogens by phagocytosis during which reactive oxygen species are generated (Paape et al., 2003).

Our study investigated whether the immune cells, involved in protecting the mammary glands from infection, exhibited increased PARP1 activity. Moreover, we evaluated whether PAR levels increased in cells isolated from milk particularly during an udder inflammation in dairy cattle. A few recent studies have reported data on PARP1 in cattle (Demeyere et al., 2013; Saccà et al., 2016) yet to our knowledge this is the first study evaluating PARP1 expression and its activity in cells purified from the blood and milk of dairy cattle. The aims of the study were (1) to characterize PARP1 expression in bovine peripheral blood mononuclear cells (PBMC) and (2) to evaluate PAR levels as a potential marker of inflammation in cells isolated from blood and milk samples following different types of infection, including mastitis.

2. Materials and methods

2.1. Animals and sample collection

The management and care of the experimental animals was carried out in compliance with the 86/609EEC European Union directive guidelines. The animals were kept at the Research Centre for Animal Production and Aquaculture of CREA where blood samples from lactating cows were collected according to good veterinary practice. Two separate trials were performed to determine PARP1 and PAR levels in cells from blood and from milk samples. In the *Trial 1,* blood samples were taken from healthy cows in order to evaluate *PARP1* mRNA (n = 5 cows) and PARP1 protein (n = 2 cows) in purified PBMC, lymphocytes and monocytes. In addition, blood samples from 2 *Staphylococcus aureus* were taken from naturally infected cows in order to determine PARP1 and PAR levels in PBMCs isolated during infection. The status of *S. aureus* infection was established by the local Institute for animal public health (Istituto Zooprofilattico Sperimentale-IZSLT). In *Trial* 2, 36 single quarter milk samples were collected separately from 9 cows in order to determine PAR levels in purified milk somatic cells. The milk cells obtained from different udder quarters were separated using the Somatic Cell Count (SCC), a parameter used as indicator of udder health and milk quality, considering a threshold of 100,000 cells/mL to define a quarter as normal (Schwarz et al. 2011; Pilla et al., 2013).

2.2. Trial 1

2.2.1. Isolation of PBMC and separation of lymphocytes and monocytes

The blood samples (50 mL) were taken from the external jugular vein and placed in vacutainer tubes containing anticoagulant EDTA and PBMC were isolated by density gradient (Lymphoprep™-1.077 g/mL; AXIS-SHIELD) according to the manufacturer's instructions. Monocytes and lymphocytes were separated from purified bovine PBMC (4×10^7 cells) by positive selection of monocytes using magnetic CD14 MicroBeads (Miltenyi Biotech) according to the manufacturer's instructions and subsequently stored at −80 °C. Furthermore, after separation aliquots of cells were stained with the mouse anti-human CD14 (clone TÜK4, Miltenyi Biotech), fluorescein isothiocyanate (FITC) conjugated antibody, and analyzed using a CytoFLEX flow cytometer (Beckman Coulter Inc). Lymphocytes and monocytes were identified in a back-gate on FSC *versus* SSC dot plot and CD14-negative and CD14-positive cells respectively. Monocytes purity was approximately 98% and lymphocytes purity was 95%.

2.2.2. Whole cell extract preparation and Western blot analysis

Aliquots of PBMC, lymphocytes and monocytes were thawed on ice and lysed immediately in RIPA buffer (50 mM Tris−HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA), supplemented with sodium-orthovanadate (3 mM), NaF (100 mM) and a mix of protease inhibitors (Sigma-Aldrich). The lysates were kept on ice for 25 min and then centrifuged (16,000*g* for 30 min at 4 °C). The supernatants were collected and quantified by the Bradford protein assay reagent (Bio-Rad). The proteins were loaded and separated by SDS-PAGE and electro-blotted onto nitrocellulose membrane (Hybond™ECL™, GE Healthcare). The immunoblots were probed with a specific PARP1 antibody (clone C2-10, Enzo Life Sciences), which recognizes full length PARP1 (116 kDa) and apoptosis-induced 89 kDa cleavage fragments, and by a specific anti-PAR antibody (clone 10H Enzo Life Sciences).

Secondary antibodies, labelled with horseradish peroxidase (HRP), were used to develop the immunoblot detection using an enhanced chemoluminescence reaction (ECL Plus, BioRad). Protein levels of the full length PARP1 band (116 kDa) were quantified densitometrically using Quantity One Software (Bio-Rad).

In order to control the specificity of anti-PAR antibody, time- dependent PAR formation was assessed in Human colon carcinoma HCT116 cells treated with the DNA damaging agent doxorubicin (Doxo) (Sigma-Aldrich). HCT116 cells were grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. When the cells were approximately 70% confluent, they were treated with 1 μM Doxo at different times up to 2 h and when indicated with ABT-888 (Sigma-Aldrich). More specifically, HCT116 cells were pre-treated with 0,5 μM ABT-888 30 min prior to Doxo treatment and the inhibitor was maintained in the medium throughout the course of the damage (2 h).

Total cell extracts were obtained by lysis in RIPA buffer, as previously described. Equal amounts of protein were separated by SDS-PAGE, electroblotted onto nitrocellulose membrane and probed with the specific anti-PAR antibody.

2.2.3. Primer design, RNA extraction and RT-qPCR

In order to detect *PARP1* gene expression, primers were designed based on the *Bos taurus* sequence (Gen Bank Accession Number: NM_174751) to amplify a 158 bp segment spanning from exon 12 to exon 13 of the bovine *PARP1* gene. The ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) and the mitochondrial ribosomal protein S15 (*MRPS15*) were tested for reference gene

Table 1
Characteristics of the primer pairs used and the efficiency of amplification.

Gene	Primer sequence (5' to 3')	Accession Number	bp	% Efficiency
PARP1	For-CATCGTCAAAGGGACCAACT Rew-AAATGCTCAATGGCATCCTC	NM_174751	158	100–104
ATP5B	For-TTTGGACTCCACGTCTCGCATC Rew-TCCTGGAGGGATTTGTAGTCCTG	NM_175796.2	108	97–102
MRPS15	For-GCAGCTTATGAGCAAGGTCGT Rew-GCTCATCAGCAGATAGCGCTT	NM_001192201.1	151	99–103