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Characterization of monoclonal antibodies generated against bovine and porcine prostacyclin synthase and quantitation of bovine prostacyclin synthase

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Abstract

Monoclonal antibodies were raised against prostacyclin synthases purified from bovine and porcine aortae, respectively. Two monoclonal antibodies, RS1 and RS2, were purified and characterized. As shown by enzyme activity precipitation and Western blot analysis, in solubilized bovine and porcine aortae microsomes the monoclonal antibodies reacted only with prostacyclin synthase. The monoclonal antibody RS1 cross-reacts with partially purified prostacyclin synthase from human umbilical veins in an ELISA-based assay. None of the antibodies inhibited the enzyme activity. By combination of the monoclonal antibody RS2 with a polyclonal antibody we established an enzyme-linked immunosorbent assay (ELISA) for quantitation of bovine prostacyclin synthase. ELISA data were confirmed by Western blot analysis. Among different bovine tissues, aortae with 1665 ± 200 ng/mg microsomal protein showed the highest content of PGIS. Significant lower concentrations were observed in tongue, lung, kidney and thymus ranging from 49 ± 13.4 to 2.7 ± 0.9 ng/mg protein. The monoclonal antibody RS1 binds to endothelial cells and vascular smooth muscle cells in human liver tissue.

Key words: Monoclonal antibody; Prostaglandin; Prostacyclin synthase; Immunohistochemistry; Immunoquantitation

1. Introduction

Prostacyclin is the most potent naturally occurring inhibitor of platelet aggregation and also a powerful vasodilator [1]. It is formed by isomerization from the prostaglandin endoperoxide PGH_2, catalyzed by the enzyme prostacyclin synthase (PGIS), a membrane-bound protein associated with the microsomal membranes. Enzymatic activity has been measured in many tissues by determination of its stable metabolite 6-keto-$PGF_{1\alpha}$. The enzyme is mainly located in mammalian blood vessels, namely in the endothelial and the smooth muscle cells [2–4]. Appreciable PGI_2 synthetic activity has also been reported for a few non-vascular cells including rabbit papillary collecting tubule cells [5], human foreskin fibroblasts [6] and bovine corpora lutea [7]. However, the cellular sites of PGI_2 synthesis have not been defined yet. Purification to homogeneity of PGIS from bovine and porcine aortae has been achieved by our laboratory [8] and others [9]. The protein was characterized by us previously as a cytochrome P_{450}-like enzyme on the basis of its spectroscopic properties [10]. Recently we succeeded in cloning and sequencing the cDNA for bovine PGIS [11].

For localization and quantitation of the enzyme in various tissues, the immunochemical approach is ideally suited. Although the isolation of monoclonal antibodies has been reported [9], only a study on the localization of the enzyme among different smooth muscle cells has been performed [12], but an assessment of the distribution and the enzyme concentration of PGIS in different organs is lacking. Furthermore no cross-reactivity to the enzyme from human sources is known. In this investigation, we describe the generation and characterization of monoclonal antibodies directed against purified bovine and porcine PGIS and their use in histochemical, Western blot analysis and immunoquantitation.

2. Materials and methods

All culture media, supplements and chemicals were obtained from sources as reported earlier [13].

2.1. Purification of prostacyclin synthase

The enzyme was isolated to homogeneity from bovine and porcine aortae as described [8]. The tissues were obtained from a local slaughter house, human umbilical veins were provided by a nearby hospital.

2.2. Culture of bovine aortic endothelial cell

Endothelial cells were isolated from bovine aortae and grown for 5–7 days in culture flasks as described [20]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Microsomes were prepared from 10^7 cells.

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; PGIS, prostacyclin synthase; PG, prostaglandin; HHD, 12-hydroxy-8,10-heptadeca-dienoic acid; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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2.3. Preparation and purification of polyclonal and monoclonal antibodies

Polyclonal and monoclonal antibodies directed to purified PGIS from bovine and porcine aortae were prepared according to a similar scheme reported recently [13]. Large amounts of monoclonal antibodies were produced by culturing hybridoma cells in roller bottles. The antibodies were purified from culture supernatants by protein G immunoaffinity chromatography according to the instruction of the supplier and stored at a concentration of 2 mg/ml at −20°C.

2.4. ELISA

The reactivity of hybridoma culture supernatants was assessed by ELISA. Purified PGIS from bovine or porcine aortae (50 ng/well) were adsorbed to microtiter plates. Residual binding sites in the wells were blocked by 0.5% gelatin in PBS. After washing, plates were incubated with hybridoma supernatants and incubated for 1 h. The wells were washed again and incubated for 1 h with peroxidase-labeled goat anti-mouse IgG. Peroxidase reaction was initiated by the addition of phenylenediamine (1 mg/ml) and 0.01% H_2O_2 and maintained for 15 min at 37°C and read at 492 nm. By this procedure several hybridoma cell lines producing anti-PGIS antibodies were obtained. Two clones were selected for further characterization.

For analysis of cross-reactivity, 200 ng/ml of partially purified enzyme (after the DEAE-purification step) from bovine and porcine aortae and from human umbilical veins were used (specific activities: 215, 44, and 3.8 nmol/mg/min, respectively).

2.5. Western blot

Western blot analyses were performed as described in a recent paper [14] except that the enhanced chemiluminescence-technique was used for visualization.

2.6. Measurement of enzyme activity

Enzymatic activity of PGIS was determined by the conversion of PGH_1 to HHD, as described by us recently [15,16]. Briefly, enzyme samples were diluted in 500 μl reaction buffer (10 mM K_2HPO_4, 20% glycerol, 1 mM EDTA, 0.2% Lubrol PX, pH 7.5) and the reaction was started by the addition of 14 μM PGH_1. The formation of HHD was monitored at 233 nm and from the initial slopes enzyme activities were calculated .

2.7. Immunoprecipitation

For precipitation of PGIS by monoclonal antibodies, enzyme samples from porcine and bovine aortae after the DEAE purification step were used. 50 μl of various dilutions of monoclonal antibodies, prebound overnight to protein G-Sepharose, was incubated with 100 μl of enzyme sample (specific activity: 215 and 44 nmol/mg/min for bovine and porcine PGIS) for 2 h on ice. The reaction tubes were centrifuged at 10,000 × *g* and the remaining enzyme activity was measured in the supernatant.

2.8. IgG subclass determination

The subclasses of the monoclonal antibodies were determined as described in [13].

2.9. Immunohistochemistry

Liver tissue was shock-frozen at −100°C and frozen sections were layered onto microscope slides, fixed by acetone for 10 min at room temperature and incubated with purified RS1 monoclonal antibody in a dilution of 1:100. The sections were washed twice and stained with fluorescein-labeled goat anti-mouse IgG_1 diluted 1:20. The sections were viewed using band pass filter selective for green fluorescence. As a negative control, the monoclonal antibody RS1 was omitted.

2.10. Immunoquantitation of PGIS

For immunoenzymometry, purified polyclonal antibodies were diluted in 50 mM $NaHCO_3$, pH 8.3, and coated onto microtiter plates for 1 h at room temperature in a humid chamber. Free binding sites were blocked by washing the plates twice for 30 min with 0.5% BSA. Solubilized microsomes (200 μg/well) or purified bovine PGIS as standard were added and incubated for a 1 h at room temperature. After washing, assays were incubated with purified monoclonal antibody RS2 (1:10.000 dilution) for 1 h. Visualization of the enzyme was performed as described in section 2.4.

3. Results

For raising monoclonal antibodies, purified PGIS from bovine and porcine tissues were injected into mice and were also used for screening of the hybridoma microcultures. Several genetically stable clones were obtained. Two of them were selected for further studies. RS1 directed against porcine PGIS and RS2 with specificity towards bovine PGIS. Both antibodies belong to the IgG_1 subclass. Large scale production was performed by cell culture and antibodies were subsequently purified by protein G-Sepharose chromatography (data not shown).

Their binding specificity is demonstrated in Fig. 1 as revealed by the ELISA technique. Serial dilutions of the monoclonal antibodies show concentration-dependent binding to the partially purified enzymes including saturability of the antigens' epitopes. RS2 raised against bovine PGIS, also possesses cross-reactivity towards porcine PGIS. However, no cross-reactivity was observed for RS1 towards bovine PGIS (data not shown). To address this phenomenon regarding human PGIS, partially purified enzyme from umbilical veins was monitored for epitope binding according to the same technique. As shown in Fig. 1, RS1 but not RS2 is cross-reactive towards human PGIS. To characterize the monoclonal antibodies under more stringent, i.e. denaturing conditions, the antibodies were examined using the immunoblot technique. Partially purified bovine and porcine protein samples were subjected to SDS-PAGE, transferred to nitrocellulose paper, and assayed for antibody staining (Fig. 2). As a control, an anti-PGIS antiserum was used. RS2 showed a single band at 52 kDa, corresponding to PGIS and excluding any other im-

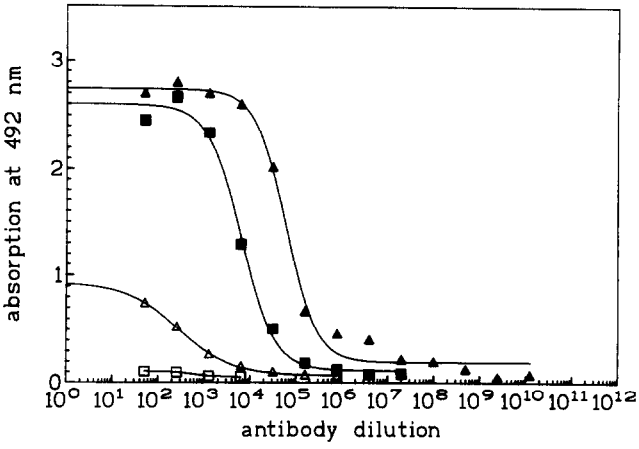

Fig. 1. Assessment of the binding capacity of the monoclonal antibodies RS1 and RS2 to partially purified PGIS from bovine and porcine aortae and from human umbilical veins. The microtiter plates were coated with 200 ng of enzyme sample. Starting from a 2 mg/ml stock solution the antibodies were used in the indicated manner Triangle = monoclonal antibody RS1; closed = porcine PGIS; open = human PGIS. Square = monoclonal antibody RS2; closed = bovine PGIS; open = human PGIS.

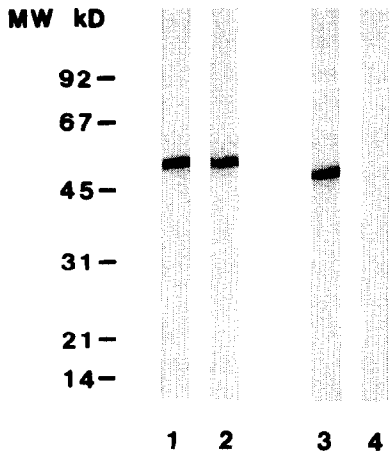

Fig. 2. Western blots of bovine and porcine solubilized microsomes probed with monoclonal and polyclonal antibodies to PGIS. DEAE-purified enzyme samples (80 μg/lane) from bovine (lane 1 and 2) and porcine aortae (lane 3 and 4), respectively, was run on 10% SDS-gels and transferred to nitrocellulose. Immunoblotting was performed essentially as described in [14]. Lane 1 and 3 = probed with polyclonal antibody; lane 2 = monoclonal antibody RS2; lane 4 = monoclonal antibody RS1.

munoreactive protein. In accordance with the results above monoclonal antibody RS1 is not reactive towards bovine PGIS after gel electrophoresis nitrocellulose transfer.

The monoclonal antibodies' specificity for PGIS was further evaluated in an immunoprecipitation assay using protein G-Sepharose for pelleting the PGIS–antibody complex. Fig. 3 demonstrates the increase of the precipitated activity as a consequence of increasing concentrations of both monoclonal antibodies. A control antibody (Tü300, a monoclonal antibody directed to thromboxane synthase [13]) could not substitute for RS1 and RS2. When protein G-Sepharose was omitted, no loss of activity was found, indicating that the monoclonal antibodies did not react with the active site of the enzymes (data not shown).

For immunoquantitation of PGIS we established a sandwich-ELISA, as we did recently for thromboxane synthase [17]. As shown in Fig. 4, the absorbance at 492 nm after log transformation was linear in the range from 20 to 800 ng. The versatility of the ELISA-based immunoquantitation was verified by difference spectroscopy of DEAE-purified PGIS (Table 1). Although these are completely different techniques, both methods revealed a similar enzyme determination.

Table 1
Determination of PGIS amount by different spectroscopy with tranylcypromine and ELISA-based immunoenzymometry

Method	Enzyme content (μg PGIS/mg protein)	Specific activity (nmol PGI_2/min/mg)
Spectroscopy with tranylcypromine*	94	1302.4
ELISA	42 ± 3.8	1302.4

PGIS enzyme preparation after the DEAE purification step was used in parallel and the enzyme content was determined as described in section 2.
*trans-2-Phenylcyclopropylamine $E_{435-414\ nm}$ = 28 $mM^{-1} \cdot cm^{-1}$.

The quantity of PGIS in different bovine tissues as calculated by the sandwich-ELISA technique is given in Table 2. A heterogenous distribution of enzyme was observed. The highest content of 1665 ± 200 ng/mg microsomal protein was found in the aortae. Much lower amounts of enzyme are present in tongue and lung, whereas it is barely detectable in kidney and thymus. Compared with microsomes from cultured bovine endothelial cells fresh bovine aortae have a nearly 10-fold higher content of PGIS. The specific activities of PGIS in the different tissues correlate well with the determined enzyme levels (Table 2).

To confirm the immunoquantitation data by Western blot, solubilized tissue microsomes (70 μg each and 20 μg for aorta) were subjected to SDS-PAGE followed by immunoblot analysis. As outlined in Fig. 5 the relative intensities of the band corresponding to purified bovine PGIS (lane 1) correlate well with the data listed in Table 2.

To demonstrate the usefulness of RS1 for immunohistochemical analyses of human tissues, monoclonal antibody RS1, which shows cross-reactivity to native PGIS from human umbilical veins, was tested on cryostat sections of human liver by immunofluorescence technique. As shown in Fig. 6, PGIS can specifically be localized in sinusoidal endothelial cells and smooth muscle cells of blood vessels. In contrast hepatocytes and Kupffer cells are devoid of staining. The photograph does not unequivally show the absence of PGIS from Kupffer cells

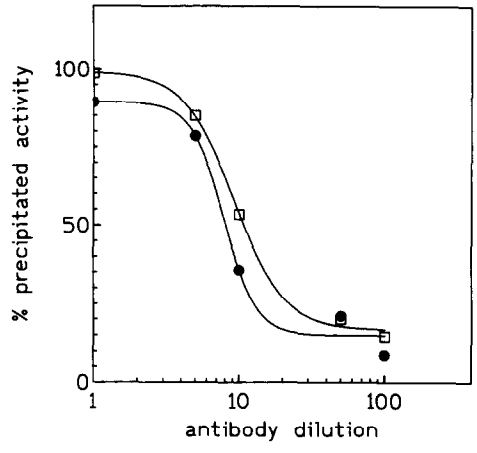

Fig. 3. Immunoprecipitation of PGIS activity by monoclonal antibodies RS1 and RS2. The assays contained partially purified enzyme from bovine or porcine aortae and monoclonal antibodies bound to protein G-Sepharose and diluted as indicated. Enzyme activities were measured in the remaining supernatant after pelleting the antibody–protein G-Sepharose–enzyme complex. Open circle = monoclonal antibody RS1; closed square = monoclonal antibody RS2.

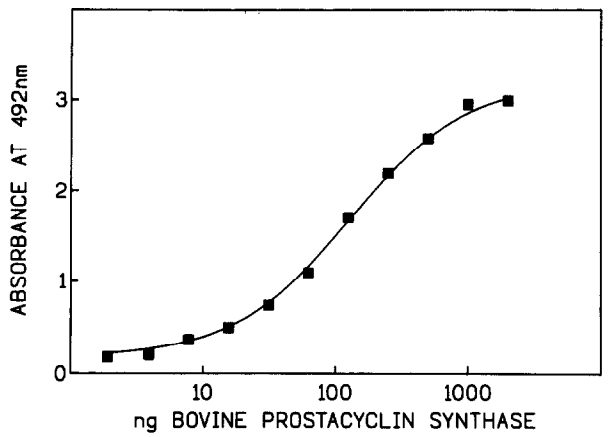

Fig. 4. ELISA standard curve for bovine PGIS. Various amounts of purified bovine PGIS were subjected to the enzyme immunoassay (circles); log transformation and linear regression (triangles). Assays were performed as described in section 2.

although available enzymological data support this assumption.

4. Discussion

The purified heme-thiolate enzyme prostacyclin synthase from bovine and porcine aortae were successfully used for the preparation of polyclonal and monoclonal antibodies. The specificities of the monoclonal antibody RS1, directed to porcine and RS2, directed to bovine PGIS, were demonstrated by different techniques, such as ELISA, immunoblot analysis and precipitation of PGIS activity. As neither the polyclonal nor the monoclonal antibodies inhibited the enzyme activity, we suggest that the active site is not affected and probably protected by a pocket form. The epitope for RS1 seems to be a conformational determinant, since it was destroyed by electrophoresis. In contrast, RS2 reacts with an epitope which is uneffected by denaturing agents.

Table 2
Immunoenzymometric assessment of prostacyclin synthase content and specific activity in solubilized microsomes of different bovine tissues

Tissue	Enzyme content (ng PGIS/mg protein)	Specific activity (nmol PGI_2/min/mg)
Tongue	49 ± 13.4	0.8
Lung	28 ± 4.1	0.7
Liver	14.3 ± 2.1	0.35
Thymus	2.7 ± 0.9	0.16
Endothelial cells	127.6 ± 48	1.2
Aorta	1665 ± 200	13.2
Kidney	8.5 ± 1.9	0.47

Both parameters were determined as described in 'Materials and Methods'. The data are mean values of 4 experiments + S.D.

One of the most promising applications of the antibodies will be for immunohistochemistry. The data demonstrate that monoclonal antibody RS1 cross-reacts with the enzyme partially purified from human umbilical veins and stains endothelial and smooth muscle cells in human liver sections. This finding agrees with reports on the capacity of these cells to synthesize prostacyclin.

A sandwich-ELISA has been developped and successfully applied in quantitating PGIS levels of different bovine tissues. Compared to an immunoradiometric assay [18], this method has the advantage to circumvent radioactive labeling without substantial loss of simplicity and sensitivity. Here we report for the first time on the PGIS enzyme content in different tissues and correlate its amount with the specific activities. The high quantity of enzyme found in aorta tissue underlines the important function of PGI_2 for hemostasis. However, compared to the aorta tissue the PGIS content in cultured bovine endothelial cells is one order of magnitude lower. These findings are in contrast to data reflecting similar amounts of PGIS in endothelial and smooth muscle cells [19]. Our results would explain the low levels of PGIS found in other tissues with a much lower portion of smooth muscle cells. But, because we measured enzyme content and activity in microsomal preparations, enzyme present in other compartments or being solubilized during microsome preparation could escape detection. For some types of smooth muscle cells an immunocytochemical study [12] reported on the association of PGIS with the cell surface and the nuclear membrane, indicating at least two separate membrane systems.

In view of the important role of prostacyclin especially as a physiological antagonist of thromboxane A_2, further cellular and subcellular localization and quantitation of PGIS will be a subject of future investigations. In this context the characterized monoclonal antibodies will be valuable tools.

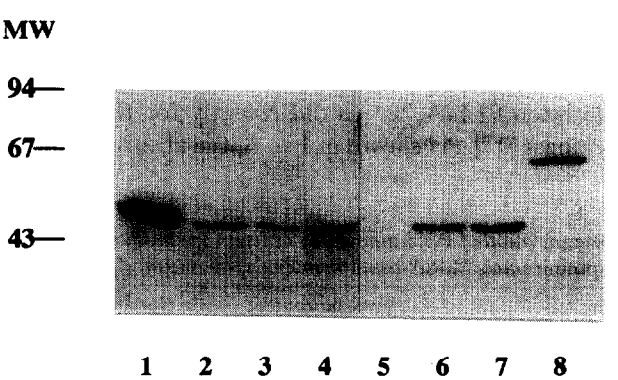

Fig. 5. Immunoblot analysis of solubilized microsomes from different bovine tissues. Solubilized microsomes (70 μg each, 20 μg for aorta) were separated by SDS-PAGE and transferred to nitrocellulose paper. Lane 1 = control; lane 2 = tongue; lane 3 = lung; lane 4 = liver; lane 5 = thymus; lane 6 = bovine endothelial cells; lane 7 = bovine aorta; lane 8 = kidney. Blotting was performed as described in section 2. As control 200 ng of purified bovine PGIS was used.

Fig. 6. Photomicrograph of a cryostat section of human liver incubated with monoclonal antibody RS1. Immunoreactivity was visualized with fluorescein-labeled anti-mouse IgG_1 antibodies as described in section 2. Magnification: 27×.

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