Isoforms of cytochrome P450 on organic nitrate-derived nitric oxide release in human heart vessels

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Abstract Glutathione S-transferases and the cytochrome P450 system have been proposed for the vascular biotransformation systems in the metabolic activation of organic nitrates. The present study was designed to elucidate the role of human cytochrome P450 isoforms on nitric oxide formation from organic nitrates using lymphoblast microsomes transfected with human CYP isoforms cDNA. CYP3A4-transfected microsomes had the most effective potential of nitric oxide formation from isosorbide dinitrate. Anti-CYP3A2 antibody (which cross-reacts with CYP3A4) or ketoconazole (an inhibitor of the CYP3A superfamily) inhibited nitric oxide formation from isosorbide dinitrate in rat heart microsomes. Immunohistochemistry of human heart also showed intense bindings of CYP3A4 antibody in the endothelium of the endocardium and coronary vessels. These results suggest that the CYP3A4-NADPH-cytochrome P450 reductase system specifically participates in nitric oxide formation from isosorbide dinitrate.

Key words: Organic nitrate; Nitric oxide; Cytochrome P450; Human heart vessel; CYP3A4

1. Introduction

Organic nitrates such as glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN) elicit vasodilatation by generating nitric oxide (NO) which activates soluble guanylyl cyclase and a cGMP-dependent effector cascade that finally leads to relaxation of vascular smooth muscles [1–5]. The enzyme(s) in vascular smooth muscle that mediate(s) the formation of NO from organic nitrates has/have yet to be identified. Formation of NO from GTN has been demonstrated in intact bovine pulmonary [6] and coronary [7] arteries, cultured porcine aortic smooth muscle cells [8] and in a broken cell preparation from bovine coronary [9] and dog carotid [10] arteries. It was demonstrated that in cell-free preparations from rat liver, cytochrome P450 (P450)-dependent enzymes as well as glutathione S-transferase (GST) may catalyze NO formation from organic nitrates [11,12]. Furthermore, it has been reported that NO formation from these compounds is mediated by a P450 system rather than by GST or free thiols in cultured RFL-6 cells [13]. These evidences were brought by the usage of either inhibitor, SKF525A, an inhibitor for global P450, or sulfobromophtalein (SBP), an inhibitor for GSTs. The unanswered questions are, however, which isoform(s) of P450 families is/are responsible for the P450-elicited organic nitrates-derived tissue NO formation. The present study was performed to demonstrate NO formation from organic nitrates in the microsomes of lymphoblasts transfected with various human CYP isoform cDNAs and which P450 isoforms exist on the human heart.

2. Materials and methods

2.1. Chemicals

Glucose-6 phosphate (G6P), glucose-6 phosphate dehydrogenase (G6PDH) and NADPH were purchased from Oriental Yeast (Tokyo, Japan). Microsomes from human lymphoblasts transfected with CYP1A2, 2A6, 2D6, 2C9, 2E1, 3A4 or 4A11 genes were obtained from Gentest (Woburn, MA, USA). Other reagents used were of analytical grade from Wako Pure Chemicals (Osaka, Japan). ISDN was a gift from Eisai (Tokyo, Japan). ISDN (100 mM) was dissolved in 100% methanol. Ketoconazole or SBP was used as an inhibitor for the CYP3A family or GSTs, respectively.

2.2. ISDN-induced NO formation in transfected human CYP isoforms

A CYP-transfected microsome preparation (100 pmol) was suspended in 0.5 ml (total volume) of phosphate-buffered saline (PBS, pH 7.4) and incubated with 3.3 mM G6P, 0.5 U/ml G6PDH, 1 mM NADPH, 3.3 mM $MgCl_2 \cdot 6H_2O$ and 200 μM ISDN for 15 min at 37°C. After incubation, samples were immediately kept on ice and centrifuged at $100\,000 \times g$ for 60 min at 4°C. Since NO released from ISDN is immediately converted to nitrite under aerobic conditions, supernatants were used for the analysis of nitrite (NO_2^-) by HPLC [14].

2.3. The effect of anti-CYP3A2, 2E1 or 2C11 antibody on ISDN-induced NO formation

Rat heart microsomes were prepared as described previously [15]. Polyclonal antibodies against P450 PB-1 (CYP3A2), CYP2E1 and P450 UT-2 (CYP2C11) were raised in rabbits and immunoglobulin G (IgG) fractions were prepared as reported previously [16,17]. Human CYP3A4 is a homologue of rat CYP3A2. We already reported that an antibody against rat CYP3A2 cross-reacted with human CYP3A4 and completely inhibited the catalytic activities of human CYP3A4 [17]. 500 μl of microsome (0.3 mg protein/ml) suspended in PBS (pH 7.4) was pre-incubated with anti-CYP isoform antibody (0.025–0.1 mg IgG) for 20 min at room temperature and subsequently with 100 μM ISDN and cofactors (G6P, G6PDH, NADPH and $MgCl_2$) for 15 min at 37°C. After incubation, samples were immediately kept on ice and centrifuged at $100\,000 \times g$ for 60 min at 4°C. Supernatants were also used for the analyses of nitrite by HPLC [14].

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Abbreviations: P450, cytochrome P450; G6P, glucose-6 phosphate; G6PDH, glucose-6 phosphate dehydrogenase; GTN, glyceryl trinitrate; GST, glutathione S-transferase; IgG, immunoglobulin G; ISDN, isosorbide dinitrate; NO, nitric oxide; NO_2^-, nitrite

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To certify CYP-induced NO release from ISDN, NO was directly measured using an NO-selective electrode (model NO-501, Inter Medical, Tokyo, Japan). Briefly, 1 ml of microsomes (0.3 mg/ml) was pre-incubated with or without anti-CYP3A2 (0.1 mg IgG) or ketoconazole (10 μM) as described above. Then, the NO electrode was carefully placed and monitored in the microsome suspension in the presence of cofactors (G6P, G6PDH and $MgCl_2$). Then, ISDN (100 μM) and NADPH (1 mM) were added successively to initiate the reaction. The obtained electrical current was recorded as previously reported [18] and a change in the electrical current was considered as an index of NO release [19]. We checked the response of NO electrodes using *S*-nitroso-*N*-acetyl-DL-penicillamine or a NO-saturated solution (1.9 mM of NO).

2.4. Immunohistochemistry of human heart vessels

Samples were obtained at the Okayama University Medical School from autopsy cases without heart failure. Cut pieces of heart samples were fixed with 10% buffered formalin and embedded in paraffin. The 4 μm thin sections deparaffinized with xylol and ethanol. They were treated with 0.3% hydrogen peroxide in methanol for the inhibition of endogenous peroxidase activity for 20 min and normal goat serum (Dako, Kyoto; diluted to 1:100) for the inhibition of non-specific binding of the second antibody for 30 min. Polyclonal antibodies for human CYP3A4, CYP2E1, CYP2C9, CYP1A1, CYP2A6 and CYP2D6 (×300) for overnight treatment at 4°C, biotin-labelled goat anti-rabbit IgG serum (Dako; diluted to 1:200) including normal serum (1:80) for 40 min and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA; diluted to 1:100) for 40 min were serially applied. The stain intensity by the bridged immunoperoxidase (peroxidase anti-peroxidase/diaminobenzidine) was checked under microscopy. And then, methyl green or hematoxylin was applied for nuclear staining. Non-immune rabbit serum in place of specific antibodies was used for the negative control. Anti-CYP isoforms with an excess fresh microsomes were pre-incubated with each antibody for overnight at 4°C. The absorbed antibody also showed negative staining.

2.5. Statistical analysis

Unless otherwise stated, data are presented as means ± S.E.M.

3. Results

3.1. ISDN-induced NO formation in transfected human CYP isoforms

The microsomal preparations from transfected lymphoblasts catalyzed NO formation, from ISDN as a substrate (Fig. 1). CYP3A4-transfected microsomes had the most effective potential of NO_2^- formation from ISDN. In contrast, CYP1A2 and CYP4A11 had little catalytic activity to generate NO_2^- from ISDN. Microsome- or NADPH-free systems did not generate NO_2^- from ISDN.

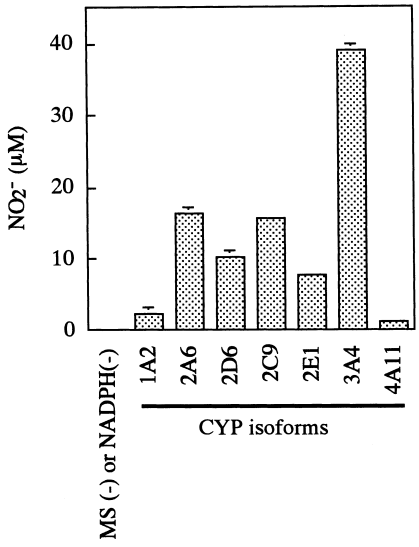

Fig. 1. ISDN-derived NO formation in human CYP isoforms-transfected microsomes as catalysts. A CYP-transfected microsome (100 pmol) was suspended in 0.5 ml of PBS (pH 7.4) and incubated with 3.3 mM of G6P, 0.5 U/ml G6PDH, 1 mM NADPH, 3.3 mM $MgCl_2$·6H2O and 200 μM ISDN for 15 min at 37°C. After incubation, samples were immediately kept on ice and centrifuged at 100 000×*g* for 60 min at 4°C. Supernatant was used for the analysis of nitrite by HPLC. Data represent means of triplicate determinations and S.E.M.

Table 1
The effect of anti-CYP isoforms antibody or a CYP3A inhibitor on NO formation from ISDN in rat heart microsomes

		% Inhibition	
		NO_2^-	NO
Control (μM)		(4.84 ± 0.22)	(5.37 ± 1.49)
anti-CYP3A2	(0.025 mg IgG)	18.3 ± 11.6	–
	(0.05 mg IgG)	44.0 ± 18.1	–
	(0.1 mg IgG)	51.9 ± 22.5	86.4 ± 28.0
anti-CYP2E1	(0.1 mg IgG)	0	0
anti-CYP2C11	(0.1 mg IgG)	0.1 ± 2.2	0
Ketoconazole	(10 μM)	64.5 ± 0.8	75.6 ± 5.5

One ml microsomes (0.3 mg protein/ml) suspended in PBS (pH 7.4) was pre-incubated with each anti-CYP isoform antibody (0.025–0.1 mg IgG) or ketoconazole (10 μM) for 20 min at room temperature and then incubated with 100 μM of ISDN and cofactors as described in Fig. 1. 15 min after incubation, samples were immediately kept on ice and centrifuged at 100 000×*g* for 60 min at 4°C. Supernatant was also used for the analysis of nitrite by HPLC. NO levels for 15 min measured by a NO electrode were calculated [18]. Data represent means of triplicate determinations and S.E.M.

3.2. Inhibitory effect of anti-CYP antibodies on ISDN-induced NO_2^- formation in rat heart microsomes

NO_2^- formation from ISDN was 4.84 ± 0.22 μM for 15 min in rat heart microsomes. Neither the microsome-free nor NADPH-free reaction mixture produced NO_2^-. Pretreatment of rat heart microsomes with anti-CYP3A2 antibody inhibited NO_2^- formation dose-dependently (Table 1) and anti-CYP2E1 or -CYP2C11 antibodies did not inhibit the NO_2^- formation. Pretreatment with ketoconazole also inhibited NO_2^- formation by about 65%. Pretreatment with SBP (100 μM) instead of antibody did not inhibit the NO formation (data not shown).

3.3. Inhibitory effect of anti-CYP3A2 or ketaconazole on ISDN-induced NO formation in rat heart microsomes

To gain direct evidence of NO release from ISDN, a NO electrode was used. Typical tracing figures are shown in Fig. 2. Addition of ISDN alone to the reaction media did not generate NO. Addition of NADPH started to produce NO formation. Pretreatment with anti-CYP3A2 antibody or ketoconazole inhibited NO biotransformation from ISDN. Total levels of NO for 15 min were shown in Table 1.

3.4. Immunohistochemistry of human heart

A list of human specimens from autopsy is shown in Table 2. Fig. 3 shows the typical immunohistochemistry of CYP3A4 in the endocardium of patient 1 (left) and the coronary artery of patient 2 (right). Intense antibody binding of CYP3A4, CYP2E1 and CYP1A2 was observed in the endothelium of the endocardium and coronary vessels. No antibody binding

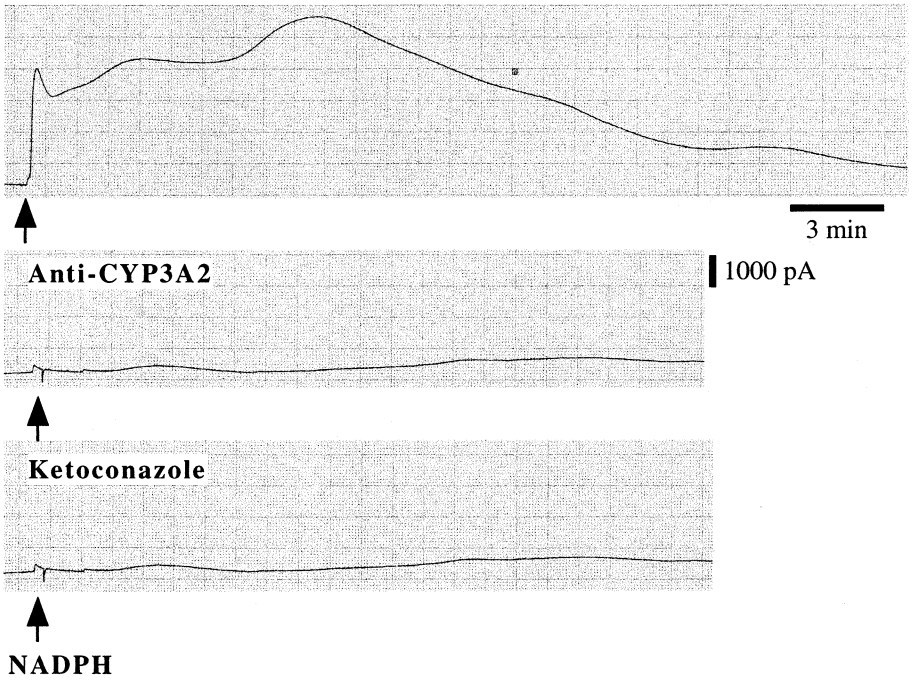

Fig. 2. Typical tracing of NO production from ISDN by CYP3A4-induced biotransformation. One ml of rat heart microsome (0.3 mg protein/ml) suspended in PBS (pH 7.4) was pre-incubated in the absence or presence of anti-CYP3A2 antibody (0.1 mg IgG) or ketoconazole (10 μM) for 20 min at room temperature and then incubated with 100 μM ISDN and cofactors as described in Fig. 1. A NO electrode was placed in the mixture before ISDN addition. Then, NADPH (1 mM) was added to initiate the reaction.

of CYP2C9 or CYP2D6 was observed. Smooth muscle cells were slightly stained by all antibodies. Other specimens from patient 3, 4 and 5 were the same as Fig. 3.

4. Discussion

We showed the participation of the CYP isoforms, especially of the CYP3A4-NADPH-cytochrome P450 reductase system, in NO formation from an organic nitrate. We also showed evidence of the localization of CYP isoforms in the human endocardium and coronary vessels.

It has been demonstrated that both GST and P450 denitrate various organic nitrates [7,12,13,20–23]. In fact, it has been reported that the P450 system and NADPH-cytochrome P450 reductase system are present in vascular smooth muscle in rabbit [24] and rat aortic microsomes possess P450 activity [22]. However, the role of P450 isoforms on human vascular site(s) of organic nitrate biotransformation has not been clarified. In this study, we found that intense antibody bindings of some CYP isoforms were observed in the endothelium of the endocardium, coronary arteries and veins. This discrepancy may be due to methodological differences between the immunohistochemistry and Western blotting of aortal microsomes and positional and species differences of vessels. Although CYP3A4, CYP2E1 and CYP1A2 were present on the vascular endothelium in human heart, CYP3A4 generated NO from ISDN more than twice that generated by other isoforms. Consistent with these results, ISDN-derived NO generation in rat heart microsomes was strongly inhibited only by anti-CYP3A2 antibody or ketoconazole (CYP3A subfamily inhibitor).

Table 2
Human specimens

Autopsy diagnosis	Age	Sex
1. Lung cancer	20	Male
2. Sepsis	76	Male
3. Hepatocellular carcinoma	59	Female
4. Malignant lymphoma	44	Male
5. Renal cell carcinoma	45	Male

A preliminary experiment also revealed that each anti-CYP3A2 antibody, but not SBP, also inhibited biotransformation of organic nitrate by the NADPH-dependent P450-P450 reductase system in human hepatic microsomes. This supports the former reports that NO formation from organic nitrates is mediated by a P450 enzyme system rather than by GSTs [7,13,25,26].

Development of tolerance to organic nitrate is a clinical problem [27,28]. The cause is not known. Organic nitrate therapy, GTN as an example, appears to be associated with a depletion of free thiols in vascular smooth muscle [29]. It is known that NO reacts with various molecules such as heme or thiols [30]. Our previous report suggested that P450, which is a heme protein, might be inactivated at the sites of the heme moiety and cysteinyl residues by NO [31]. When CYP3A4 plays a major role in the NO release from organic nitrates as this paper describes, generated NO may partially inhibit the catalytic activity of this isoform (and others), which will result in a decrease of NO generation from organic nitrates. This may be related to development of tolerance of organic nitrates. Consistent with our notions (formation of nitrosothiol(s) of P450), pronounced activation of a partially purified human soluble guanylate cyclase by GTN was observed after the addition of cysteine [32]. This mechanism might be due to reversed active sites of P450 cysteinyl residues by cysteine.

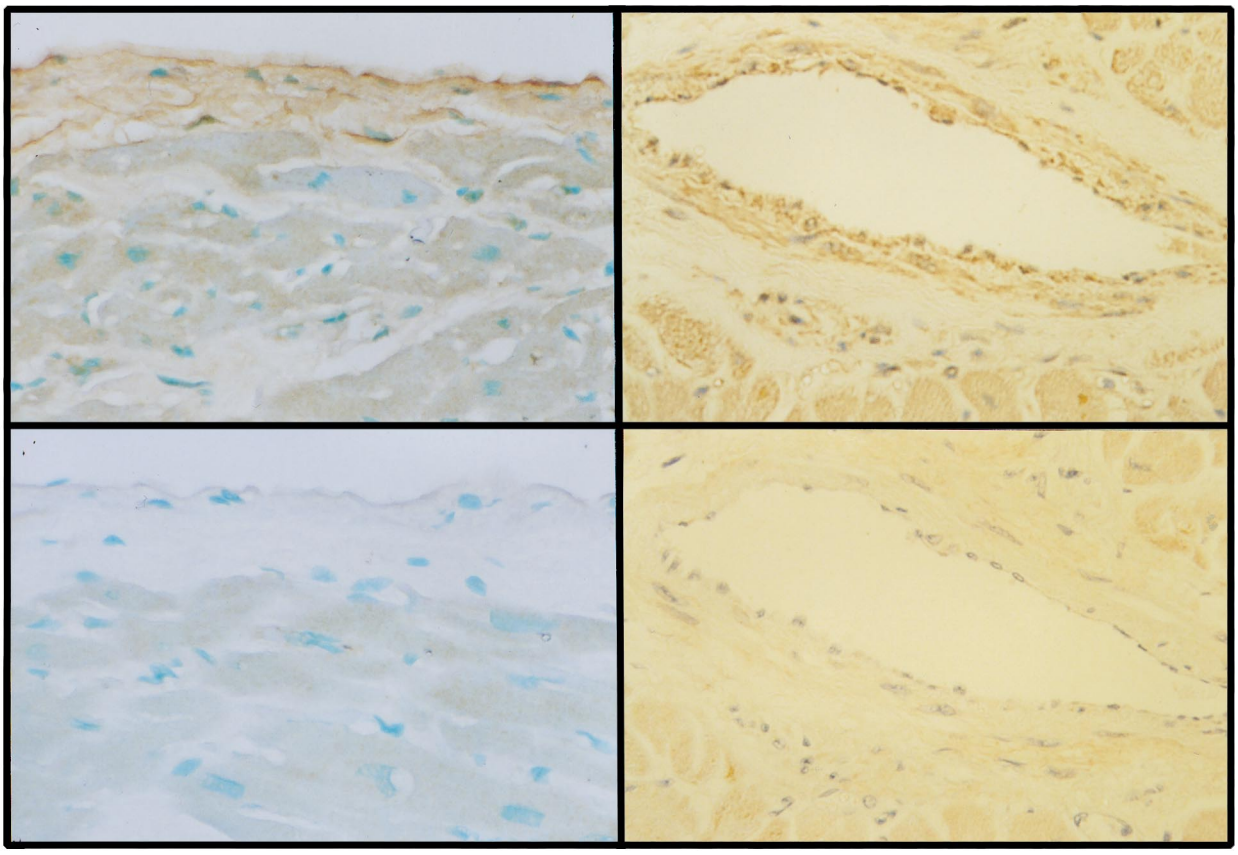

Fig. 3. Immunohistochemistry of human CYP3A4 in the heart. Specimens were obtained from an autopsy case 1 (left) and case 2 (right). Upper panel, anti-CYP3A4; below panel, normal rabbit IgG, original magnification (×400).

In summary, we have provided strong and direct evidence for CYP3A4-mediated biotransformation of organic nitrates in human heart vessels.

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