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Stress-Specific Responses of p21 Expression: Implication of Transcript Variant p21 alt-a in Long-Term Hypoxia

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ABSTRACT

p21 (CDKN1A, Cip1, Waf1) is a cyclin-dependent kinase inhibitor capable of causing cell cycle arrest or promoting cell cycle transit as well as acting as a regulator of apoptosis. In this study, we analyzed the effects of various antemortem conditions on p21 protein level and expression profiles of known p21 transcript variants in human heart tissue. The selected death cause groups were: non-cardiac, hypothermia, acute ischemia, and chronic hypoxia. Immunohistochemical staining of p21 in cardiac myocytes could be observed only in hypothermia death cases, in which the mRNA expression of the most abundant variant, p21V1, also exceeded that in other death cause groups. Cytoplasmic localization of p21 protein in vascular smooth muscle cells together with substantially increased expression of cardioprotective Pim-1 especially in chronic hypoxia, but in acute ischemia and hypothermia as well, indicate change of p21 function from cell cycle arrest to promotion of proliferation and cell survival in these cases. In chronic hypoxia deaths the expression of variant p21 alt-a was highly pronounced whereas the expression of variant p21B was low. In chronic hypoxia deaths the expression of p53 was substantially higher compared to the other groups, being a potential regulator of p21 alt-a expression. In acute ischemia deaths increased expression of variant p21B, suggested to be proapoptotic in several cell lines, was observed. Our results suggest a role for variant p21 alt-a in hypoxia and for variant p21B in acute myocardial ischemia. The known cardioprotective aspect of hypothermia might come from an increased p21 protein level. J. Cell. Biochem. 113: 544–552, 2012.

KEY WORDS: p21; HYPOXIA; HYPOTHERMIA; CELL CYCLE TRANSIT; APOPTOSIS; CARDIOPROTECTION

The heart is composed of differentiated cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts accompanied by cardiac progenitor cells (CPCs). The adult human heart has traditionally been considered to be a postmitotic organ, although myocardial smooth muscle cells, endothelial cells, and fibroblasts are able to proliferate. Some cardiomyocyte proliferation in damaged myocardium has been reported as well [Kajstura et al., 1998, Beltrami et al., 2001]. More recent studies have suggested that the ability of CPCs to differentiate to other cardiac cell lineages could be the source of new cardiomyocytes—both in physiological and pathological situations [Kajstura et al., 2008]. The efficiency of these renewal events is, however, still under debate [Kajstura et al., 2010ab; Bergmann et al., 2011].

Tissue homeostasis is maintained by balanced regulation of cell proliferation, differentiation, and cell death. Regulation of cell cycle progression is essential in various cellular events, and the cell cycle has a number of checkpoints that are needed for appropriate cell division. A universal cyclin-dependent kinase inhibitor, p21 (CDKN1A, Cip1, Waf1), is a negative regulator of growth and its overexpression can induce G1/S or G2/M cell cycle arrest [Cayrol et al., 1998]. DNA damage results in p53-dependent transcriptional activation of p21, and in addition, several transcription factors act on p21 in a p53-independent way [Gartel and Tyner, 2002].

Cell death is a process that can be triggered by diverse cellular events in health and disease. Redundant or damaged cells are excised either by necrosis or apoptosis. The regulation of apoptosis,

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programmed cell death, has been intensively studied. Recent evidence has been presented for the multiple and somewhat conflicting roles of p21 as a modulator of apoptosis through different mechanisms [Jänicke et al., 2007]. The significance and role of p21 in apoptosis is far from being clear.

Eight different p21 splice variants have been identified in human [el-Deiry et al., 1994; Nozell and Chen, 2002; Radhakrishnan et al., 2006]. Seven of these variants, p21V1, p21V2, p21C, p21 alt-a, p21 alt-a', p21 alt-b, and p21 alt-c, encode for protein p21. Variant p21B encodes for an entirely different protein, p21B. The promoters for p21 and p21B contain common response elements for transcription factors, whereas some response elements are found exclusively from the p21 promoter [Nozell and Chen, 2002]. This allows simultaneous expression of both proteins or expression of only one of the two proteins. p21 is post-translationally regulated by various serine and threonine kinases, which leads to cell- and context-dependent dissociation of p21 from proliferating cell nuclear antigen (PCNA), translocation of p21 from nucleus to cytoplasm and stabilization or degradation of the protein [Sheaff et al., 2000; Rössig et al., 2001; Zhou et al., 2001].

Phosphorylation of p21 by serine and threonine kinase Pim-1 stabilizes the otherwise short-lived protein [Sheaff et al., 2000] and induces its shift from nucleus to cytoplasm [Wang et al., 2002]. This change in cellular localization alters the function of p21 from cell cycle arrest to proliferation, or to either pro- or antiapoptotic pathway [Zhou et al., 2001; Jänicke et al., 2007]. Pim-1 has been shown to act cardioprotectively by regulating cell proliferation and inhibiting apoptosis of cardiomyocytes in cell culture and animal studies [Muraski et al., 2007; Borillo et al., 2010]. This cardioprotection proved to be independent of the activation state of Akt, an upstream effector of Pim-1 [Muraski et al., 2007]. Increased CPC cycling has also been suggested as one of the possible mechanisms of cardioprotection by Pim-1 [Cottage et al., 2010]. In cardiac explants from human donors Pim-1 has been shown to be upregulated at both protein and mRNA level in failing heart myocardium, and similar results were obtained from a mouse model of chronic heart failure. Pim-1 was proposed to reduce infarction injury and apoptosis in the myocardium [Muraski et al., 2007].

Deprivation of oxygen causes tissue injury, and hypoxia-induced cardiomyocyte apoptosis is believed to be responsible for part of the cell loss in heart failure [Sabbah et al., 1998]. In cultured cardiomyocytes increased activity and protein accumulation of p53 as well as increased expression of p53 target p21 in hypoxia-induced apoptosis have been observed [Long et al., 1997]. Furthermore, in hypoxia-induced apoptosis of cardiomyocytes increased BAX/Bcl-2 mRNA ratio has also been demonstrated [Yang et al., 2008]. Already mild hypothermia is known to cause cardiovascular changes, and when temperature decreases, bradycardia and eventually the risk of ventricular fibrillation and asystole increases [Mallet, 2002]. At the same time, hypothermia is known to generate myocardial protection, which is believed to originate from decreased metabolism and oxygen consumption [Vinas et al., 1979]. Accumulating evidence shows that hypothermia has an effect on several cell survival and apoptotic pathways [Ning et al., 2007; Hsu et al., 2009; Yang et al., 2009].

In this study, we investigated the expression of p21 at protein and transcript levels in myocardial autopsy samples from deceased persons with cardiac or non-cardiac causes of death. Divergent gene expression should arise from activation of variable regulatory systems in different stresses. Our hypothesis was that antemortem hypoxia and hypothermia contribute to the expression of p21 protein and to the expression profile of p21 variant mRNAs. Since the cellular localization of p21 is postulated to affect its function, we analyzed the mRNA expression of constitutively active Pim-1. The relative expression of p53 mRNA, BAX/Bcl-2 mRNA ratio, hypoxia-inducible factor HIF1α mRNA as well as HIF1α protein level was also quantified.

MATERIALS AND METHODS

AUTOPSY CASES

Formalin-fixed paraffin-embedded heart tissues were obtained from the archives of the Institute of Diagnostics, Department of Forensic Medicine, University of Oulu, Finland. The medico-legal autopsies had been performed during the years 1991–2009 and all the subjects had died in the province of Oulu. Fresh heart tissue samples were collected at autopsies for research purposes. Transmural tissue samples originated from the left anterior wall of the heart. The collection and use of autopsy material from human subjects was approved by the National Supervisory Authority for Welfare and Health (Valvira), Finland, and the Ethics Committee of the University of Oulu, Finland.

Cases were randomly selected from the autopsy material, which was prearranged based on autopsy reports and causes of death concluded by forensic pathologists. The groups selected for the study were: (1) non-cardiac deaths (including accidental traumatic injuries, alcohol intoxications, accidental and suicidal drug intoxications, homicides and asphyxiation), (2) hypothermia deaths, (3) acute ischemia deaths (atherosclerotic heart disease with autopsy observations of acute myocardial infarction or structural heart defect), and (4) chronic hypoxia deaths (atherosclerotic heart disease with hypoxia-related obstructive lung diseases and/or autopsy observations of previous infarction).

IMMUNOHISTOCHEMISTRY

A total of 60 cases from the archives were included in the study involving 47 men and 13 women, aged 51.3 ± 15.7 (SD) years, range 13–86 years. The samples consisted of 13 non-cardiac deaths, 32 hypothermia deaths including 3 hypothermia-related deaths, 7 acute ischemia deaths and 8 chronic hypoxia deaths. The postmortem time for all specimens was between 1 and 2 days (Table I).

Heart tissue specimens were sectioned at a thickness of 5 μm. Sections were deparaffinized and rehydrated prior to automatic epitope retrieval by the PT-link system (DAKO, Glostrup, Denmark). Immunohistochemical staining was carried out in an automated instrument (Autostainer Plus, DAKO) according to standard procedures and manufacturers' instructions. Monoclonal anti-human p21 antibody (mouse IgG_{2A}; R&D Systems, Minneapolis, MN) recognizing the p21 protein was used at concentration of 8 μg/ml. 3.3'-Diaminobenzidine (DAB) (DAKO) was used as a

TABLE I. Cases in Immunohistochemistry Study

Group	Cases	Ages (years)
Non-cardiac	4 women, 9 men	39.2 ± 16.0 13–63
Hypothermia	8 women, 24 men	52.4 ± 16.0 15–86
Acute ischemia	7 men	59.0 ± 9.6 44–74
Chronic hypoxia	8 men	60.5 ± 5.2 56–70

Values are mean ± SD and range.

chromogen and slides were counterstained with hematoxylin (Reagena, Toivala, Finland). As a negative control, monoclonal mouse IgG_{2A} isotype control antibody (R&D Systems) at concentration of 8 μg/ml was used. p21 and IgG_{2A} antibodies were applied to sequential sections of specimens of each death cause group. As an endothelial cell marker monoclonal anti-human antibody CD141 (mouse IgG_{2A}; AbD Serotec, Oxford, UK) was used at 1:30 dilution. p21 staining was divided into two categories: negative (absent staining to weak staining intensity) and positive (moderate to strong staining intensity) except for staining of VSMC cytoplasm, where the intensity of staining was graded on a scale from 0 to 3. Slides were observed with a Nikon Microphot SA microscope and photographed with a Nikon FX35DX camera (Nikon, Tokyo, Japan).

REAL-TIME QUANTITATIVE RT-PCR

A limited number of fresh heart tissues were available for gene expressions studies. The samples analyzed (n = 12) consisted of 3 cases defined as non-cardiac deaths, 3 hypothermia deaths, 3 acute ischemia deaths, and 3 chronic hypoxia deaths. Postmortem times of the cases varied between 1 and 3 days (Table II).

Total RNA was extracted from tissues stored at −70°C with Eurozol (EuroClone, Lugano, Switzerland) reagent and further purified with RNA Clean up (Macherey-Nagel, Düren, Germany) according to manufacturers' instructions. The quality of RNA was evaluated electrophoretically. High Capacity cDNA RT kit (Applied Biosystems, Foster City, CA) was used to reverse transcribe the RNAs with random primers according to the manufacturer's protocol.

The complementary DNAs were amplified three to four independent times with a Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) using gene-specific primers (Sigma, Haverhill, UK) and the Maxima SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD) real-time PCR system according to the manufacturers' protocols. The cDNAs were diluted 1:20 for amplification of an endogenous reference gene, GAPDH. The amplification was carried out as follows: 1 cycle for denaturation (95°C 10 min) followed by 40 cycles for three-stage PCR (95°C 15 s, 61.5°C 30 s, and 72°C 30 s). Fluorescence signals were measured continuously during repetitive cycles. Target gene expressions were normalized to reference gene GAPDH using the $2^{-\Delta\Delta Ct}$ method.

TABLE II. Cases in Quantitative PCR and HIF1α Protein Studies

Group	Cases	Ages (years)
Non-cardiac	1 woman, 2 men	42.7 ± 22.9 17–61
Hypothermia	2 women, 1 man	51.0 ± 36.0 15–87
Acute ischemia	3 men	55.0 ± 6.1 51–62
Chronic hypoxia	3 men	58.7 ± 4.0 55–63

Values are mean ± SD and range.

Commercial human heart total RNA (Clontech, Mountain View, CA) pooled from normal hearts of 3 Caucasian males (ages between 30 and 39 years; trauma as the cause of death) was used as a reference sample. Relative expressions of p21 variants, HIF1α, Pim-1, and p53 mRNAs were obtained by comparing the normalized expression of each target to that of corresponding target in the reference sample, which was given the value 1. Relative BAX/Bcl-2 mRNA ratios were calculated by comparing each mRNA ratio to that in the reference sample, which was given the value 1. In order to clarify relative amounts of different p21 variants, all other normalized expression values were compared to that of p21V1 in the reference sample, which was given the value 1.

PRIMERS FOR qPCR

Primers for all eight known p21 transcripts, Pim-1, HIF1α, p53, BAX, Bcl-2, and GAPDH were designed. Primer sequences for transcripts p21V1, p21V2, p21 alt-a, p21B, Pim-1, HIF1α, p53, BAX, Bcl-2, and GAPDH with corresponding GeneBank Accession numbers and amplicon sizes are shown in Table III.

PROTEIN SLOT BLOTTING

Heart tissue (Table II) homogenates were prepared using PBS buffer and a TissueLyser LT homogenizer (Qiagen, Hilden, Germany). After centrifugation the pellets were lysed with Lysis buffer (Pierce, Rockford, IL) supplemented with Halt Protease Inhibitor Cocktail (Pierce) according to the manufacturer's instructions. The protein concentrations of the lysates were measured with a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA). Equal amounts of proteins were blotted onto Hypond-P PVDF membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), using a Minifold II blotting apparatus (Whatman International, Maidstone, UK), and the membrane was probed with monoclonal anti-human/mouse/rat HIF1α antibody (R&D Systems) at 1 μg/ml concentration. HIF1α protein was visualized by standard enhanced chemiluminescence (ECL) with reagents purchased from Sigma-Aldrich (St. Louis, MO) using a Fuji LAS-3000 Luminescent Image Analyzer (Fuji, Tokyo, Japan), and quantified with the QuantityOne software (BioRad, Hemel Hempstead, UK).

STATISTICAL ANALYSIS

Statistical analyses were done using the SPSS 18 program. Immunohistochemical staining was analyzed by one-way ANOVA with a significance level of 0.05. Where group differences were confirmed, post-hoc procedure Games–Howell was used for multiple comparisons. In mRNA and Slot Blot protein expression studies the statistical analyses of the differences between means were performed pair-wise with one-way ANOVA. The findings were regarded as statistically significant when $P < 0.05$.

TABLE III. Primer Information

Gene/variant	Primer sequence	GeneBank accession number	Amplicon (bp)
p21V1	F: 5'-TCAGAGGAGGCGCCATGT-3' R: 5'-GCCATTAGCGCATCACAGT-3'	NM_000389.3	130
p21V2	F: 5'-ACAGGTGTTTCTGCGGCA-3' F: 5'-AGTCGCGGCTCAGCTGCT-3'	NM_078467	124
p21 alt-a	F: 5'-CTGTTTTCAGGCGCCATGT-3' R: 5'-GCCATTAGCGCATCACAGT-3'	BQ940274	131
p21B	F: 5'-GTGGGGTTCAATACTACAGCACAG-3' R: 5'-GCTGTATCCTGGTGGTTAAGCA-3'	Z85996	159
Pim-1	F: 5'-GTCCAAAATCAACTCGCTTGC-3' R: 5'-CACCTGGTACTGCGACTCCA-3'	NM_002648.3	112
HIF1α	F: 5'-CCGCTGGAGACACAATCATA-3' R: 5'-TGCAGGGTCAGCACTACTTC-3'	NM_181054.2	200
p53	F: 5'-TGGTGCCCTATGAGCCGCCTG-3' R: 5'-GCCGCCCATGCAGGAACTGTT-3'	NM_001126117.1	86
BAX	F: 5'-CGGTGCCTCAGGATGCGTC-3' R: 5'-ACGGCGGCAATCATCCTCTG-3'	NM_ 004324.3	103
Bcl-2	F: 5'-GAGGCTGGGATGCCTTTGTGGA-3' R: 5'-AAGCTCCCACCAGGGCCAAACT-3'	NM_000633.2	108
GAPDH	F: 5'-TGGAAGGACTCATGACCACA-3' R: 5'-TTCAGGTCAGGGATGACCTT-3'	BC029618	160

RESULTS

p21 PROTEIN EXPRESSION IN CARDIAC MYOCYTES

In hypothermia death group, positive p21 immunostaining of cardiac myocyte cytoplasm (Fig. 1A, asterisk) was detected in 44% of the cases, which was clearly higher than in other groups ($P \leq 0.001$ vs. non-cardiac and acute ischemia deaths). A considerable number of nuclei were also positively stained in 59% of the cases in hypothermia deaths (Fig. 1A, circle; hypothermia deaths vs. acute ischemia deaths $P \leq 0.001$). Nuclear p21 staining was observed especially in large nuclei. Although some of the hypothermia death cases had been exposed to freezing and thawing, the nuclei seemed intact and no cell shrinkage or extension of the extracellular space was detected on microscopic examination. Only faint non-specific immunostaining in some cases could be seen with negative isotype control antibody (Fig. 1B)(Table IV).

Non-cardiac (Fig. 2A), acute ischemia (Fig. 2B) and the majority of chronic hypoxia (Fig. 2C) deaths showed negative p21 immunostaining of cytoplasm of cardiac myocytes. Only one positively stained case was seen in the chronic hypoxia group. Nuclear myocyte staining was observed in 23% of the cases in non-cardiac deaths and in 13% of the cases in chronic hypoxia deaths, while in acute ischemia death cases no nuclear myocyte staining could be seen (Table IV).

p21 PROTEIN EXPRESSION IN VASCULAR CELLS

VSMCs of blood vessel walls showed positive cytoplasmic p21 immunostaining in all non-cardiac (Fig. 2A), acute ischemia (Fig. 2B) and chronic hypoxia (Fig. 2C) deaths, and in most of cases in hypothermia (Fig. 1A) deaths. The mean value for staining was slightly higher in chronic hypoxia deaths compared to other deaths; however, there was no statistical difference between the groups. In 16% of hypothermia death cases occasional positive nuclear p21 staining of VSMCs in the innermost layer of blood vessels was observed (Fig. 1A, arrow) (Table IV). No p21 immunostaining of vascular endothelial cells could be seen, while as a control, positive staining for endothelial cell marker CD141 is shown in Figures 1C and 2D–F.

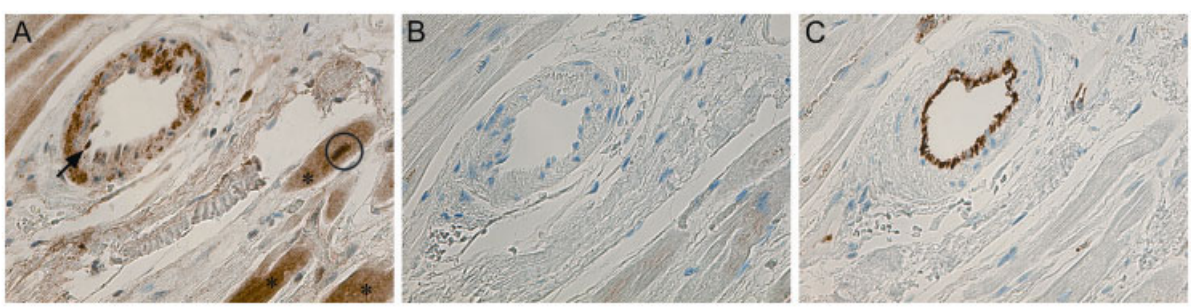

Fig. 1. Immunohistochemistry of hypothermia death heart tissues. p21-staining of cardiac myocyte cytoplasm (A; asterisk) and nuclei (A; circle) is shown. Immunoreactivity can be seen in VSMC cytoplasm (A) and in some of the VSMC nuclei in the innermost layer of arterial blood vessels (A; arrow). Hypothermia death heart tissue with isotype IgG_{2A} control antibody as a negative control (B) and CD141 antibody as a marker for vascular endothelial cells (C). Original magnification 200×. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE IV. Summary of p21 Immunostaining Results

A	Myocyte cytoplasm	Myocyte nuclei	VSMC nuclei	B VSMC cytoplasm
Non-cardiac	0% (0/13)	23% (3/13)	0% (0/13)	100% (13/13) 1.8 ± 0.6
Hypothermia	44% (14/32)*	59% (19/32)	16% (5/32)	97% (31/32) 2.0 ± 0.5
Acute ischemia	0% (0/7)	0% (0/7)	0% (0/7)	100% (7/7) 1.7 ± 0.8
Chronic hypoxia	13% (1/8)	13% (1/8)	0% (0/8)	100% (8/8) 2.3 ± 0.5

A: Values are percent positive and total positive/total number of cases in parenthesis.
B: Values are percent positive, total positive/total number of cases in parenthesis and means of staining intensity $\pm$ standard deviation (SD).
*$P \leq 0.001$ compared with non-cardiac deaths.

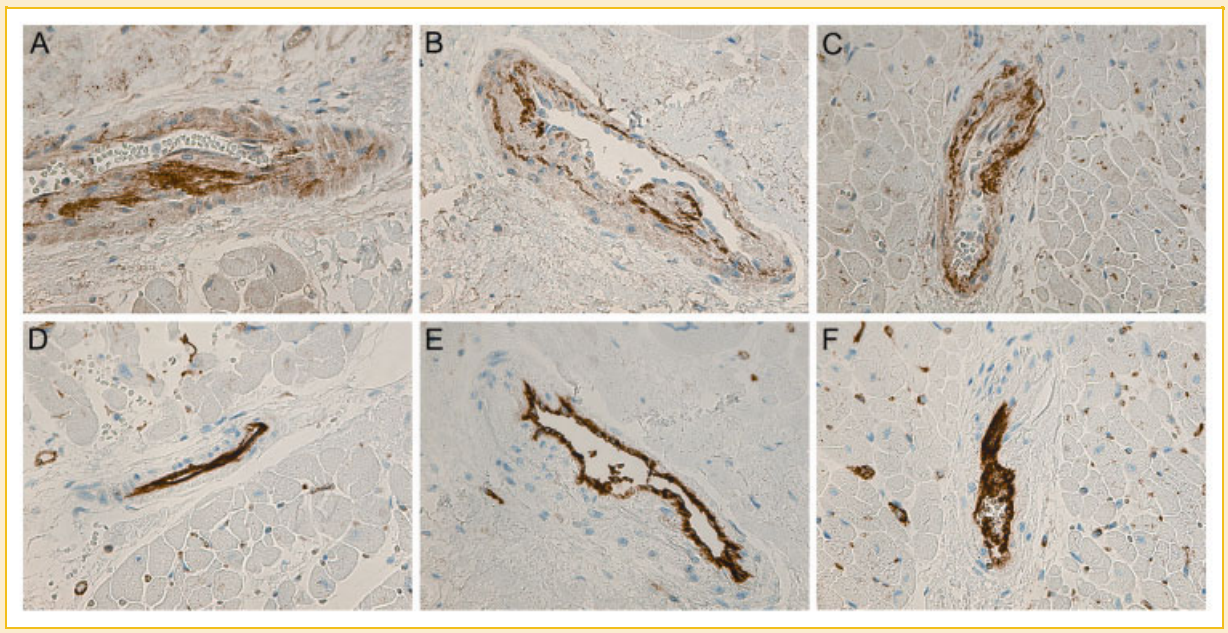

Fig. 2. p21 immunostaining of non-cardiac (A), acute ischemia (B), and chronic hypoxia (C) death heart tissues. Only cytoplasmic staining of VSMCs can be detected. Endothelial marker CD141 staining in non-cardiac (D), acute ischemia (E), and chronic hypoxia (F) deaths can be seen. Original magnification 200×. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

EXPRESSIONS OF p21 VARIANT mRNAs IN DIFFERENT DEATH CAUSE GROUPS

Figure 3 shows electrophoresis of total RNA samples. RNAs were partially degraded in various degrees. However, ribosomal 18S and 28S RNA bands were visible in all samples, and RNAs were suitable for the method used in this study. Partial degradation of RNA has been shown to have no influence on the real-time qPCR when the amplicons are short (70–250 bp) and the expressions are normalized to an endogenous reference gene [Fleige and Pfaffl, 2006]. Relative expressions of p21 variant mRNAs are illustrated in Table V.

The biggest change in relative expressions was a 62-fold increase for variant p21 alt-a in chronic hypoxia deaths ($P \leq 0.05$ vs. non-cardiac deaths, hypothermia deaths and acute ischemia deaths). A more modest 7-fold increase for p21 alt-a was observed in acute ischemia deaths ($P \leq 0.01$ vs. non-cardiac deaths) as well. The relative expression of p21V2 in chronic hypoxia deaths was 9-fold increased ($P \leq 0.05$ vs. non-cardiac deaths and acute ischemia deaths) (Table V).

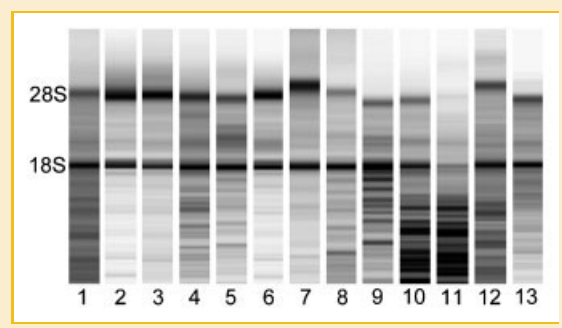

Fig. 3. Electrophoresis of extracted heart RNAs. Lane 1: Commercial human heart total RNA, lanes 2–4: non-cardiac deaths, lanes 5–7: hypothermia deaths, lanes 8–10: acute ischemia deaths, and lanes 11–13: chronic hypoxia deaths. Positions of 18S and 28S ribosomal RNA indicated in lane 1. RNAs were analyzed separately, and therefore the distance migrated by 18S and 28S varies between samples.

TABLE V. Relative Expressions of p21 Variants

	p21V1	p21V2	p21 alt-a	p21B
Non-cardiac deaths	1.0 ± 0.6	1.9 ± 0.8	1.9 ± 1.7	≤0.9† ± 0.8
	0.4–1.6	1.0–2.5	0.4–3.7	0.1–1.7
Hypothermia deaths	5.1 ± 3.1	5.1 ± 4.9	3.4 ± 1.1	5.7* ± 3.0
	2.0–8.2	1.1–10.6	2.5–4.6	3.1–8.9
Acute ischemia deaths	4.0 ± 2.4	2.9 ± 1.5	6.5** ± 0.5	20.5** ± 7.3
	1.5–6.2	1.2–3.8	6.1–7.0	12.1–25.6
Chronic hypoxia deaths	1.9 ± 0.2	9.4* ± 3.7	62.2* ± 33.4	≤0.3† ± 0.1
	1.8–2.1	5.9–13.2	31.8–98.0	0.2–0.4

The data shown are mean ± SD and range. If the mRNA expression was too low to be detected during 40 cycles of qPCR, Ct value 40 was used in calculations. The more accurate value of relative expression may be lower than reported.
†For two of the three cases value 40 was used in 1–2 measurements.
*$P \leq 0.05$.
**$P \leq 0.01$ compared with non-cardiac deaths.

In acute ischemia deaths the relative expression of p21B had a substantial 20-fold increase ($P \leq 0.01$ vs. non-cardiac deaths), while the expression of p21B in chronic hypoxia deaths was decreased (no statistical significance vs. non-cardiac deaths; $P \leq 0.01$ vs. acute ischemia). In hypothermia deaths a 6-fold increase could be detected for variant p21B ($P \leq 0.05$ vs. non-cardiac deaths). Expression of p21B in hypothermia deaths differed from expression in acute ischemia ($P \leq 0.05$) and chronic hypoxia ($P \leq 0.05$) deaths as well (Table V).

In hypothermia deaths the expression of variant p21V1 was 5-fold and variant p21 alt-a 3-fold increased ($P \leq 0.01$ vs. acute ischemia and $P \leq 0.05$ vs. chronic hypoxia). The mean increase for p21V2 in hypothermia group was 5-fold, but there was great variation from case to case, and no statistical significance between groups could be found. Similar divergence could also be seen in acute ischemia deaths in the relative expression of p21V1 (Table V).

The mean changes in the relative expressions in non-cardiac deaths were small. One of the cases placed into this group based on the primary cause of death had autopsy findings showing chronic bronchitis and pulmonary emphysema. The relative expression of p21 alt-a (3.7) of this case clearly exceeded the mean expression value (1.1) for the other cases in this group (Table V).

Relative expression for variant p21alt-c varied greatly from case to case, with no detectable correlation to different death cause groups (data not shown). Due to the shortness of dissimilar sections in sequences, it was not possible to design primers specific for variants p21 alt-a' and p21 alt-b. We tried to analyze p21 alt-a and p21 alt-a' simultaneously, but only p21 alt-a could be amplified. In the case of p21C, we were not able to detect an amplicon specific for this variant.

RELATIVE AMOUNTS OF THE p21 VARIANT MRNAS

Figure 4 shows variant p21V1 to be the major variant in all death cause groups. The relative amount of p21 alt-a in chronic hypoxia deaths was comparable to that of p21V1. The relative amounts of p21 alt-a in the other death groups and p21B and p21V2 in all death groups was minor compared to those mentioned above (Fig. 4).

PIM-1, HIF1α1, AND p53 mRNA EXPRESSIONS AND BAX/BCL-2 mRNA RATIO IN DIFFERENT DEATH CAUSE GROUPS

The relative expression of Pim-1 mRNA was substantially increased in all groups, with the highest value in chronic hypoxia deaths (230 ± 105; $P \leq 0.05$ vs. non-cardiac deaths, hypothermia deaths and acute ischemia deaths). In non-cardiac deaths one substantially higher value (34.1) for the expression of Pim-1 raised the mean value (13.6). Without this case the mean value would be 3.4. This was the case with the divergent p21 alt-a expression level. The value of the same case for HIF1α expression (2.3) and for p53 expression (18.1) also deviated from the other cases in this group, raising the mean value from 0.9 to 1.4 and from 2.3 to 7.6, respectively. The mean relative mRNA expression of HIF1α was, however, close to 1 in all other death cause groups except for acute ischemia deaths, in which the mean relative expression was approximately 2-fold higher (no statistical significance). The mean relative expression of p53 in chronic hypoxia deaths was 7–14-fold higher than in the other death cause groups (103.3 ± 25.0; $P \leq 0.01$ vs. non-cardiac deaths, hypothermia deaths and acute ischemia deaths) (Table VI).

The relative BAX/Bcl-2 mRNA expression ratio was very similar in non-cardiac, hypothermia and acute ischemia deaths, and slightly higher in chronic hypoxia deaths ($P \leq 0.05$ vs. non-cardiac deaths) (Table VI).

HIF1α PROTEIN EXPRESSION

No statistical differences between the mean relative HIF1α protein levels in various death cause groups could be detected. Only two cases of non-cardiac deaths could be included in the protein expression study due to lack of sufficient amount of tissue (Fig. 5).

DISCUSSION

Adaptation of cells to diverse stresses leads to specific p21 mRNA transcript variant profiles in cell culture conditions [Radhakrishnan et al., 2006; Millau et al., 2009]. The majority of studies on molecular mechanisms in the heart, both in health and disease, have utilized cell cultures, animal experiments, or diseased organs obtained from heart transplantation patients. The use of autopsy material with different causes of death gives both protein and transcript level information that is otherwise unattainable.

Appropriate delivery of oxygen is crucial for cardiac gene expression. Hypoxia-inducible factor (HIF) is the major transcription factor induced in tissues as a response to decreased oxygen concentration. Expression of the HIF1α gene is upregulated by the onset of hypoxia, and increased HIF1α mRNA expression is an early response to myocardial ischemia or infarction. In normal ventricular heart tissue or in heart with signs of old infarction, the level of HIF1α mRNA expression is low [Lee et al., 2000]. Our cardiac death subgroups coincided with these findings, the mean relative expression of HIF1α being 2-fold increased in acute ischemia deaths, and staying at the level of the reference sample in chronic hypoxia deaths. In our study we were not able to show differences in protein levels of HIF1α in our death cause groups.

The most striking observation in p21 variant expression profiles was the highly increased expression of p21 alt-a in chronic hypoxia

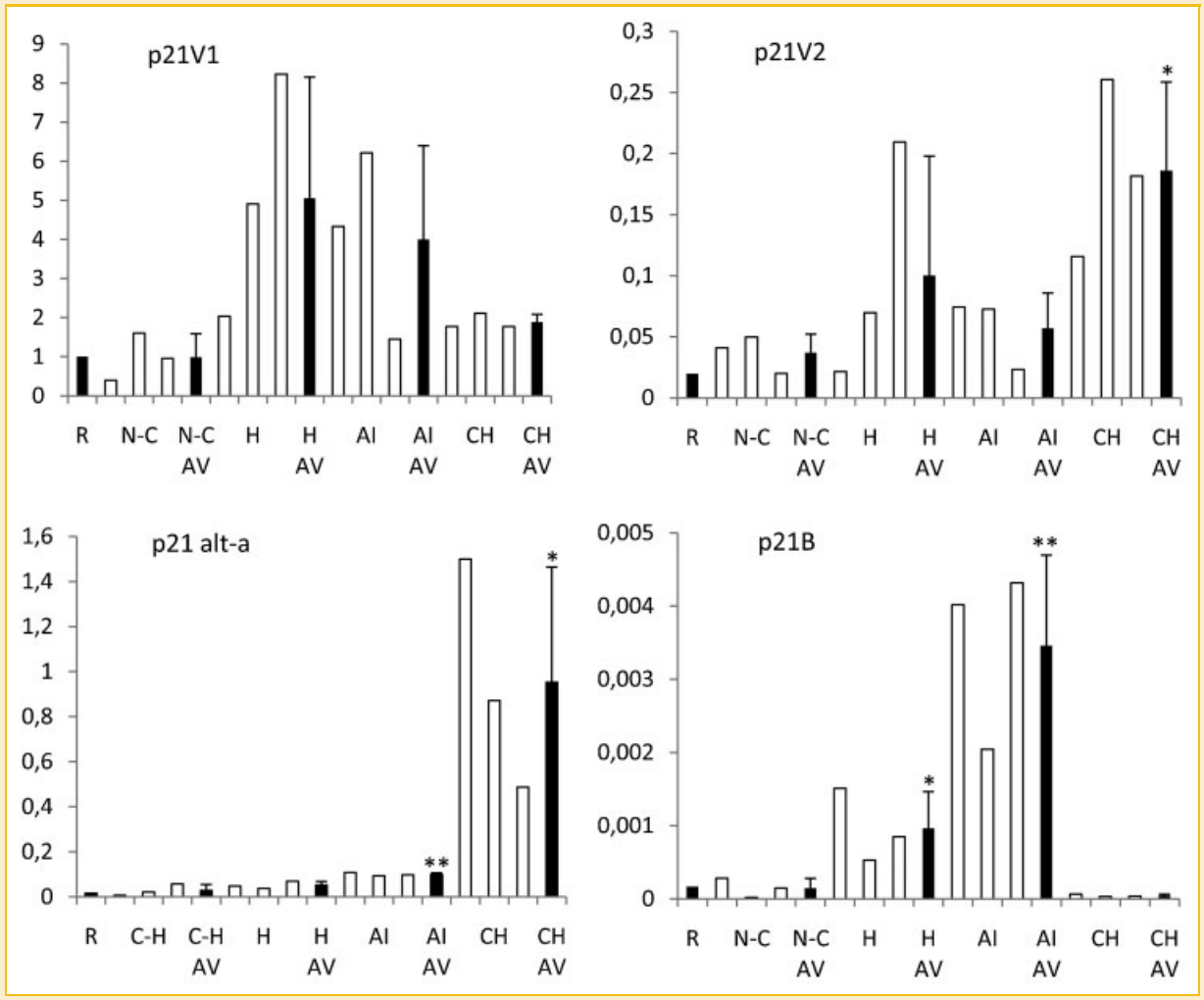

Fig. 4. Relative amounts of p21 transcript variants in different death cause groups. Normalized expression values (three cases in each death cause group) of all variants were compared to that of p21V1 in commercial reference sample, which was given the value 1. Abbreviations used: R, reference sample; N-C, non-cardiac deaths; H, hypothermia deaths; AI, acute ischemia deaths; CH, chronic hypoxia deaths; AV, mean ± SD; and $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared with non-cardiac deaths.

deaths. Autopsy reports of these cases evidenced long-term hypoxia. In acute ischemia deaths there was also a clear increase in the relative expression of p21 alt-a, however, not nearly as pronounced as in chronic hypoxia deaths. Further suggestion for a relation between lower oxygen level and expression of this variant was the case in non-cardiac deaths with hypoxia-associated autopsy findings and higher relative expression of p21 alt-a compared to the other cases in the same group. p21 gene contains several p53 response elements, and different stresses result in specific p53-binding patterns and thereby in diverse p21 variant expression profiles [Millau et al., 2009]. One of the p53 response elements is located just upstream of putative transcription start site of p21 alt-a variant. In chronic hypoxia deaths the relative expression of hypoxia induced p53 mRNA was clearly higher compared to other death cause groups. Based on these results, our conclusion is that an increase in p21 alt-a expression is probably p53-dependently connected to the duration and degree of hypoxia in the heart.

Increase in p21 alt-a expression in the heart could be one mechanism for adaptation to hypoxia. Increase in tissue vascularization improves delivery of oxygen, and chronic hypoxia has been shown to lead to higher myocardial arteriolar density [Tian et al., 2009]. Induced transcription of endogenous p21 and elevated

TABLE VI. Relative Pim-1, HIF1α and p53 mRNA Expressions, and the Relative Ratio of BAX and Bcl-2 mRNA Expressions

	Pim-1	HIF1α	p53	BAX/Bcl-2
Non-cardiac	13.6 ± 17.7 3.3–34.1	1.4 ± 0.8 0.9–2.3	7.6 ± 9.1 1.8–18.1	0.95 ± 0.02 0.93–0.97
Hypothermia	40.2 ± 17.4 22.8–57.6	1.2 ± 0.5 0.8–1.8	11.6 ± 6.2 4.4–17.8	0.96 ± 0.02 0.94–0.98
Acute ischemia	30.3 ± 6.5 23.0–35.3	2.3 ± 1.2 1.6–3.6	14.1 ± 8.7 4.2–20.5	0.96 ± 0.03 0.93–0.99
Chronic hypoxia	229.9* ± 105.0 114.4–319.5	1.1 ± 1.0 0.2–2.2	103.3** ± 25.0 84.2–131.6	1.01* ± 0.03 0.98–1.00

The data shown are mean ± SD and range.
$^{*}P \leq 0.05$.
$^{**}P \leq 0.01$ compared with non-cardiac deaths.

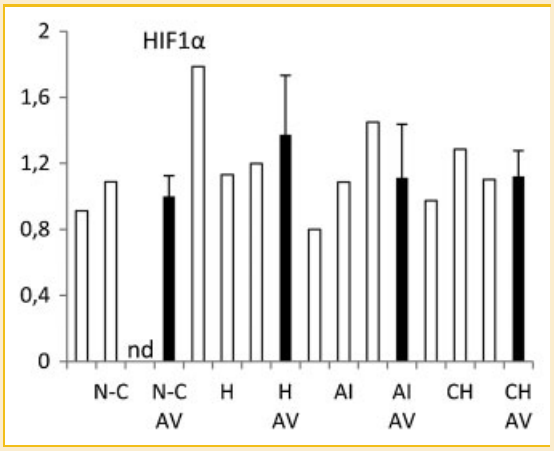

Fig. 5. Relative HIF1α protein levels in various death cause groups. Signal intensities were compared to the mean value of that of N-C cases, which was given the value 1. Abbreviations used: N-C, non-cardiac deaths; H, hypothermia deaths; AI, acute ischemia deaths; CH, chronic hypoxia deaths; AV, mean ± SD; and nd, not determined.

protein level both have a mitogenic and an anti-apoptotic role in cultured VSMCs [Zhang et al., 2003]. Studies on VSMCs have also shown that forced cytoplasmic location of p21 leads to increase in cell cycle transit [Dong et al., 2004]. Except for some hypothermia death cases with nuclear p21 staining, in our heart tissue samples the p21 protein in VSMCs could only be seen in the cytoplasm. The cytoplasmic location was most probably caused at least partly by increased expression of constitutively active Pim-1, which has also been suggested as having a role in VSMC proliferation [Katakami et al., 2004]. Our proposal is that in VSMCs, p21 alt-a functions as a promoter of hypoxia-induced cell proliferation, protection against apoptosis and concomitant increase of vascularization in the heart.

Another distinctly increased expression in chronic hypoxia deaths was observed for p21V2. A larger number of cases are needed to clarify whether this is specific for the group, since occasional increased expression values could be seen in other death cause groups as well.

The role of p21 in apoptosis is on the whole quite complex. Increased levels of p21 can both protect against apoptosis and promote apoptosis, depending on cell type, circumstances [Gartel and Tyner, 2002] and cytoplasmic location of p21 [Asada et al., 1999]. Studies on cancer cell lines have proposed a proapoptotic role to p21B [Nozell and Chen, 2002]. Regulated cell death is known to occur in cardiac myocytes of diseased hearts, but the role of regulated and non-regulated cell deaths varies depending on the type of disease. In acute myocardial infarction, large numbers of apoptotic cardiomyocytes are found in heart tissue [Whelan et al., 2010]. In our study the relative expression of the variant coding for protein p21B was substantially increased in acute ischemia deaths compared to the other groups. Based on these observations, p21B-induced apoptosis could be characteristic to acute ischemia death cases. However, BAX/Bcl-2 mRNA ratio, which reflects the apoptotic state of cells, was not increased in this group. Further more; in chronic hypoxia deaths the relative expression of p21B mRNA was decreased, while BAX/Bcl-2 ratio was increased compared to all other death groups. Whether cells survive or undergo apoptosis is known to be affected by the severity of hypoxia [Greijer and van der Wall, 2004]. No commercial antibody for p21B protein is available, and therefore the existence and cellular location of p21B protein could not be evaluated.

In some cell lines hypothermia has been demonstrated to induce an increase in mRNA and the protein level of p21 and to cause cell cycle arrest [Matijasevic et al., 1998; Ohnishi et al., 1998]. In our study, the number of hypothermia death cases expressing a detectable level of p21 protein in cardiac myocyte cytoplasm and nuclei clearly exceeded that in the other groups. Since p21V1 was the most abundant variant, its increased mRNA expression can be expected to have led to the higher protein concentration in hypothermia cases. In animal studies hypothermia has been reported to promote expression of proteins involved in cell survival of cardiomyocytes during ischemia together with inhibition of apoptosis [Ning et al., 2007]. Cytoplasmic location of p21 protein in cardiac myocytes is consistent with the cell survival role of hypothermia. However, further studies are needed to prove the relevance of cellular localization of p21 in cardiomyocytes during hypothermia induced cell survival. Nuclear p21 immunostaining in cardiac myocytes was especially seen in large, seemingly polyploidic nuclei. Polyploidization, an increase in chromosome number, has been detected during increased load on myocardium [Herget et al., 1997]. Production of polyploid cells relies on cells' ability to tolerate incomplete DNA replication. In mammals, prevention of apoptosis in polyploid cells is connected to stimulation of p21 expression [Ullah et al., 2009]. In some hypothermia deaths p21 protein could also be seen in VSMC nuclei.

In conclusion, our study suggests that different strategies employed by the heart in response to different stresses modify p21 transcript variant expression levels and profile. These results suggest that increased expression of variant p21 alt-a has an important role in hypoxic heart. Increased relative expression of variant p21B appears to be characteristic for certain types of antemortem events witnessed in acute ischemia, and to a lesser extent in hypothermia. Increased p21 protein level could be a sign of cardioprotective mechanisms in hypothermia. These results contribute to the main idea of our work to identify transcript variants connected to specific pathological conditions to be used in death cause investigations as well as prognostic disease markers.

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