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Chronic regulation of the expression of gap junction proteins connexin40, connexin43, and connexin45 in neonatal rat cardiomyocytes

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Abstract

Gap junction channels form the basis of intercellular communication in the heart. In the working myocardium, the connexin43 (Cx43) is most abundantly found, whereas connexin40 (Cx40) is expressed in the atria and in the conduction system [together with low levels of connexin45 (Cx45)]. However, little is known about the differential regulation of the connexins by pathophysiologically stimuli such as tumor necrosis factor α (TNFα). Inasmuch as TNFα may play a contributory role in the concert of factors involved in the pathophysiology of heart failure and because this cardiac disease often leads to ventricular reentrant arrhythmia, the goal of our study was to find out whether TNFα may influence the expression of the cardiac connexins connexin43, connexin40, and connexin45.

Neonatal rat cardiomyocytes were exposed to TNFα (10, 40, 100, 400, and 1000 pg/ml) for 24 h with or without additional treatment with the mitogenic-activated protein kinase (MAP-kinase) inhibitors SB203580 [4-(4-fluorophenyl)-2-(4-methyl-sulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; 10^{-5} M, protein38 mitogenic-activated protein kinase (p38 MAP kinase) inhibitor] or the MEK1 (mitogenic-activated protein kinase/extracellular signal-regulated kinase kinase) inhibitor PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; 10^{-5} M]. Connexin43, connexin40, and connexin45 expressions were analysed using Western blot analysis, immunohistology, and polymerase chain reaction (PCR) studies (connexin43 and connexin40). TNFα induced a concentration-dependent increase in connexin43 (by 2.9±0.6, $P<0.05$, $n=5$) but not in connexin40 or connexin45 expressions. Both connexins (40 and 45) showed a very low expression near the detection limit. The increases in connexin43 expression could be completely suppressed by SB203580 (0.9±0.4, $P<0.05$, $n=5$) but not by PD98059. In absence of a stimulating drug, these inhibitors (SB203580 or PD98059) did not affect connexin43 content. Additional PCR experiments revealed increases in connexin43 mRNA under the influence of 100 pg/ml TNFα (211±38%, $P<0.05$, $n=5$), which could be completely suppressed by SB203580. In contrast, the connexin40 expression remained unchanged. From these results, we conclude that TNFα can differentially regulate cardiac connexin expression via p38 MAP kinase pathway and thus may alter intercellular communication. This may contribute to the changes observed in heart failure with regard to the formation of an arrhythmogenic substrate.

Keywords: Gap junction; Connexin; Cardiomyocyte, rat; TNFα; MAP-kinase

1. Introduction

Gap junction channels, intercellular low-resistance pathways, allow intercellular communication by electrical and metabolic coupling between neighbouring cells (Bruzzone et al., 1996). The gap junction channel is composed of two hexameric hemichannels of each neighbouring cell, and the hemichannels itself consist of certain proteins, the connexins, a family of proteins with

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more than 15 members. In mammalian hearts the main connexins expressed are connexin37 (Cx37), connexin40 (Cx40), connexin43 (Cx43), and connexin45 (Cx45) (Verheule et al., 1997). It has been shown that connexin43 is the predominant connexin in ventricular cardiomyocytes, whereas Cx40 is predominantly localised in atrial cardiomyocytes and in the conduction system. The gap junction protein connexin45 was found in embryonic stages of the heart and in small amounts in Purkinje fibres (Dhein, 1998a; Gros and Jongsma, 1996; Kanter et al., 1993a,b). Connexin37 in the heart is exclusively expressed in endothelial cells. The gap junction channels formed by different connexins differ in their biophysical properties, i.e., regulation, conductivity, and permeability. In the heart, the gap junction channels allow the complex interaction between stimulus conduction and heart muscle contraction. In many diseases such as myocarditis, septicaemia, cardiac infarction, or cardiomyopathy, this interaction may be disturbed leading to life threatening arrhythmia or to depression of myocardial contractile function. There is evidence that tumor necrosis factor α (TNFα), as a proinflammatory cytokine, is somehow involved in the pathogenesis of cardiovascular diseases such as heart failure, atherosclerosis, viral myokarditis, etc. (for review, see Meldrum, 1998). However, it should be noted that clinical trials using TNFα antagonists, such as etanercept (Mann et al., 2004) or infliximab (Chung et al., 2003), have failed to show therapeutic efficacy. While according to these findings the role of TNFα in heart failure may be questionable, it is unclear whether this cytokine, which is reported to be enhanced in heart failure (see Von Haehling et al., 2004), may be involved in the accompanying remodelling process regarding gap junction remodelling. Several authors found different responses to TNFα treatment depending on the cell type (endothelial cells, microglia, and cardiomyocytes), and cytokine concentration used no influence (connexin43 and connexin32), down-regulation of several connexins (connexin32, connexin37, and connexin40), or up-regulation of connexins (connexin26, connexin43) (Eugenin et al., 2001; Fernandez-Cobo et al., 1998, 1999; Temme et al., 1998; Van Rijen et al., 1998). Thus, in different cell types, obviously different signalling pathways connected to connexin regulation are activated in response to TNFα-receptor stimulation.

Therefore, the aim of the present study was to investigate the effect of chronic TNFα stimulation on expression of the cardiac predominant gap junctions connexin40, connexin43, and connexin45. For that reason, we used cultured neonatal rat cardiomyocytes, and dose–response studies of TNFα application were done. In a second series of experiments, because several authors reported an interaction between TNFα and mitogenic-activated protein kinases (MAP-kinases; Feuerstein and Young, 2000; He et al., 1999), additional treatment with MAP-kinase inhibitors was performed.

2. Methods

2.1. Cell culture

Cardiomyocytes were isolated and cultured according to the following protocols (Polontchouk et al., 2001a): ventricles of newborn Wistar rats were digested in collagenase II solution, centrifuged, and after a preplating period to remove noncardiac cells, resuspended in M199 medium containing 2 mM L-glutamine, 100 mg/ml streptomycin and penicillin, 1% foetal calf serum, and 10% horse serum (to inhibit fibroblast growth). The cells were seeded in Petri dishes coated with 0.1% gelatine, and medium was changed three times a week. After reaching confluence, the cells were exposed to different concentrations of TNFα with or without MAP-kinase inhibitors for 24 h and subsequently were analysed using immunoblotting, immunohistology, or polymerase chain reaction (PCR). TNFα was used in concentrations between 10 and 1000 pg/ml. These concentrations were chosen according to blood TNFα levels, which can be reached in patients suffering from sepsis (200–500 pg/ml; Grau et al., 1989; Kwiatkowski et al., 1990; Perkins et al., 2000). For inhibition of the protein38 mitogenic-activated protein kinase (p38 MAP kinase), the inhibitor SB203580 [4-(4-fluorophenyl)-2-(4-methyl-sulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole] for inhibition of the MEK1 (mitogenic-activated protein kinase/extracellular signal-regulated kinase kinase), the inhibitor PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one] was used both in a concentration of 10 μM. PD98059 and SB203580 in these concentrations are specific inhibitors for MEK1 or p38 MAP kinase, respectively (Alessi et al., 1995; English and Cobb, 2002; Goedert et al., 1997). SB203580 at a concentration of 10 μM has been shown to inhibit p38 by 98%, p38β2 by 90%, while other kinases, such as MKK1 or ERK2, were inhibited only by 1% or 15%, respectively. All other kinases are inhibited by less than 15%, except that some inhibition of LCK (by 58%), GSK3β (by 34%), and PKBα (by 38%) at 10 μM has also been described (see Davies et al., 2000). The latter kinases (LCK, GSK3β, and PKBα) are not known to be involved in Cx43 expression regulation.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

In total, six series of experiments were performed:

(1) control without treatment (n=5);
(2) control+SB203580 10 μM (n=5);
(3) control+PD98059 10 μM (n=3);
(4) TNFα dose–response curve without inhibitors (n=5);
(5) TNFα dose–response curve with 10 μM SB203580 (n=5); and
(6) TNFα 100 pg/ml with 10 μM PD98059 (n=3).

After 24-h treatment, cells were lysed for 6 h at 4 °C using a hypotonic medium containing Triton-X100 and protease inhibitors [K_2HPO_4 20 mM pH 7.9, EDTA 1 mM, aprotinin 10 μg/ml, leupeptin 0.5 mg/ml, pepstatin A 7 μg/ml, Triton-X100 1%, phenylmethylsulphonyl fluoride (PMSF) 1 mM], the cells were centrifuged (5 min at 500 g), and total protein concentration was determined in the supernatants according to the method described by Lowry et al. (1951). Thereafter, Western blot analysis of the connexin40, connexin43, or connexin45 content was carried out.

In a second series of experiments for investigation of connexin43 and connexin40 mRNA, confluent monolayers of cardiomyocytes were either stimulated with TNFα (100 pg/ml) with or without additional 10^{-5} M SB203580 (n=5 each) for 24 h. For RNA isolation, cells were harvested using TRIZOL (Gibco BRL, Germany), and cDNA was synthesised by reverse transcription using 1 μg total RNA. Polymerase chain reaction (PCR) was carried out with primer pairs flanking connexin43, connexin40, or the housekeeping-gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The products of expected size (connexin43, 600 bp; connexin40, 216 bp; and GAPDH, 415 bp) were visualised in ethidium-bromide-stained 1.5% agarose gels.

In a third set of experiments, cardiomyocytes were grown on glass cover slips coated with 0.1% gelatine and treated with TNFα with or without SB203580 or PD98059 in the same manner as described above. After fixation with ice cold methanol, immunofluorescence labelling for connexin43 was performed as described by Dhein (1998b).

2.2. Western blots

The cell lysates were mixed with gel-loading buffer according to Laemmli, following classical protocols (Findlay, 1990), and for electrophoresis, 30 μg protein per slot of each protein sample was fractionated through a 4% stacking and 10% running sodium dodecyl sulfate (SDS)-polyacrylamide gel.

Proteins where then transferred electrically (semi dry blot) on to a nitrocellulose membrane and blocked with 6% low-fat milk blocker at 4 °C overnight. Primary antibody to connexin40, connexin43, or connexin45 diluted 1:500 was applied for 2 h at room temperature. Cross reactivity of these antibodies with other connexins was checked, and we found no cross reactivity as also reported in a previous study (Polontchouk et al., 2001b). Thereafter, the blots were washed three times with phosphate-buffered saline (PBS; containing NaCl 137 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM, and 0.1% Tween-20) and were incubated with secondary horseradish peroxidase-labelled antibody diluted 1:1000 for 1 h at room temperature. Thereafter, connexins were detected using the iodophenol/luminol system by application of the ECL Western blot detection kit from Amersham Pharmacia Biotech. Immunoblots were incubated in iodophenol/luminol reaction mixture (60 s) and exposed to X-ray film (10–20 min) to detect chemiluminescence. The connexin bands were imaged on a scanner, and the pictures were digitised and analysed with BioRad software (Bio-Rad, München, Germany). After background subtraction, grey scale values of connexin signals in experimental preparations (series 2–6, see above) were compared with signals of the untreated control cells (series 1), which were normalised to a value of 1.0 (according to Pimentel et al., 2002).

2.3. Reverse transcription and polymerase chain reaction (PCR) amplification

Confluent cell monolayers of neonatal rat cardiomyocytes were harvested, and RNA was isolated using TRIZOL (Gibco). Thereafter, RNA was reverse transcribed from 1 μg total RNA with random hexamer to generate fist-strand cDNA, using standard protocols (Arensbak et al., 2001). After first-strand cDNA was prepared, 1 μl cDNA was put together with PCR reagents to make a 25-μl solution containing 1U Taq DNA polymerase (Gibco), 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.5 μl of each primer. The amplification was carried out using the following primer according to Salameh et al. (2003): connexin43 antisense 5′-TTG TTT CTG TCA CCA GTA AC-3′, sense 5′-GAT GAG GAA GGA AGA GAA GC-3′, connexin40 antisense 5′-TCC CGT TCA CCT CTT TCC AG-3′, sense 5′-CCT TCC CCA TCT CCC ACA TT-3′, GAPDH antisense 5′-CCG CCT GCT TCA CCA CCT TCT-3′, sense 5′-GTC ATC ATC TCC GCC CCT TCC-3′. The cDNA amplification products of connexin43 were predicted to be 600 bp; of connexin40, 216 bp; and of GAPDH, 415 bp, respectively. The products were visualised and analysed in ethidiumbromide-stained 1.5% agarose gels.

2.4. Immunofluorescence staining

Confluent monolayers of cardiomyocytes cells were fixed in ice cold methanol (−20 °C, 10 min) and permeabilised in PBS+0.1% Triton-X for 30 min at room temperature. After an application of PBS+1% bovine serum albumin (BSA) for 20 min to block unspecific background, primary connexin43-antibody (1:100, 2 h) was applied, and after a washing step with PBS+1%, bovine serum albumin cells were incubated with secondary fluorescein isothiocyanate-labelled goat antimouse antibody (FITC-labelled antibody, Sigma, St. Louis, USA; 1:300) for 1 h in the dark. Thereafter, the monolayers were washed extensively with PBS and embedded with Karion F.

The histologic specimens were viewed through a microscope (Zeiss Axioskop, Zeiss, Jena, Germany) at 1000× magnification, and photographs were taken.

2.5. Statistical analysis

The concentration–response curves have been analysed for C_{max}, EC_{50}, and Hill slope and were fitted to a sigmoidal

curve using GraphPadPrism software (GraphPad Software, San Diego, CA, USA). A two-factorial analysis of variance (ANOVA) was performed. If ANOVA indicated significant differences, the data were additionally analysed with the Tukey HSD test. For statistical analysis, "Systat for Windows, ver. 5.02" (Systat, Evanston, USA) software was used.

2.6. Materials

TNFα and SB203580 were obtained from Alexis Biochemicals (San Diego, CA). MEK1 inhibitor PD98059 was purchased from Cell Signalling (New England Biolabs). Polyclonal rabbit anticonnexin40, monoclonal mouse anti-connexin43, and polyclonal rabbit anticonnexin45 primary antibody were bought from Chemicon (Temecula, USA). Secondary antibody horseradish or FITC-labelled antibody, M199, and all other chemicals used were obtained from Sigma (Deisenhofen, Germany). Foetal calf serum and collagenase II were purchased from Gibco Life Technologies (Karlsruhe, Germany). The primers were synthesised by BioTeZ (Berlin, Germany).

3. Results

3.1. Western blot analysis

To determine whether TNFα application can modify connexin expression, neonatal cardiomyocytes were stimulated with increasing concentrations of TNFα (10, 40, 100, 400, and 1000 pg/ml) for 24 h, and expression of connexin40, connexin43, and connexin45 was measured by immunoblotting. As shown in Fig. 1A,B TNFα caused a significant ($P<0.05$) increase in total connexin43 amount in a dose-dependent and saturable manner. The EC_{50} value calculated from the dose–response curve was 9.36±0.49 pg/ml, R^2=099. E_{max} was 2.9 optical density (OD) units and was reached at TNFα concentrations higher than 100 pg/ml. The Hill slope of the concentration–response curve of the connexin43 expression was 1.0.

In contrast to connexin43, the connexin40 expression could not be stimulated by TNFα. As seen in Fig. 2A,B, connexin40, which gives only a weak signal, is not up-regulated by application of this cytokine and stays at control levels. The third connexin examined, connexin45, only showed very low expression at the detection limit, which was also not altered by TNFα.

In a second series of experiments, the inhibitor SB203580 (10 μM) was applied simultaneously with TNFα. The additional application of the p38 MAP kinase inhibitor resulted in a significant ($P<0.05$) and complete suppression of the TNFα-induced increase in connexin43 (Fig. 1A). In contrast, the amount of connexin40 and connexin45 remained unchanged, as was observed with TNFα alone.

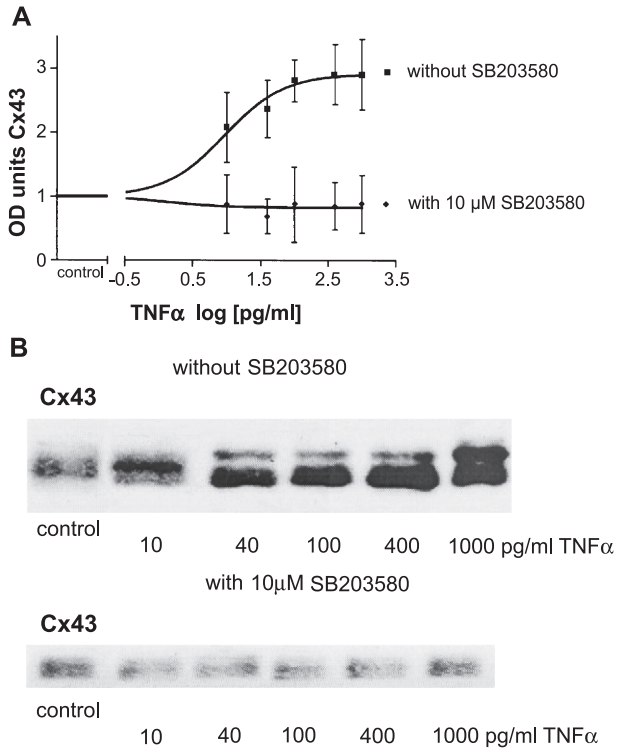

Fig. 1. (A) Dose–response curve: Western blot results for connexin43 in neonatal rat ventricular cardiomyocytes exposed for 24 h to increasing concentrations of TNFα (10, 40, 100, 400, and 1000 pg/ml) in absence or presence of the p38 MAP kinase inhibitor SB203580. All values are given as means±S.E.M. of n=5 experiments in optical density (OD) units relative to untreated control cells (see Methods). (B) Original Western blots for connexin43 in neonatal ventricular cardiomyocytes exposed for 24 h to increasing concentrations of TNFα (10, 40, 100, 400, and 1000 pg/ml) in absence or presence of the p38 MAP kinase inhibitor SB20358010.

In subsequent experiments, a possible additional effect of an MEK1 pathway was elucidated. Therefore, a single dose of 100 pg/ml TNFα (which is a submaximum concentration near saturation) was applied to the cardiomyocytes, and another MAP-kinase inhibitor, the specific MEK1-inhibitor PD98059, was administered concomitantly at a concentration of 10 μM. In contrast to the previously described experiments, the MEK1-inhibitor did not influence the increase in connexin43 content induced by TNFα (Fig. 3).

In control experiments, the influence of SB203580 and PD98059 alone on connexin expression was investigated; SB203580 and PD98059 did not exert any effects on connexin content (Fig. 3). Figs. 1B and 2B show representative Western blots as a compilation of the experiments.

3.2. PCR analysis of Cx43-expression

The PCR analysis of connexin43 mRNA revealed similar findings, as described above on the protein level. Thus, we found a significant increase in connexin43 mRNA relative to the housekeeping gene GAPDH mRNA under the

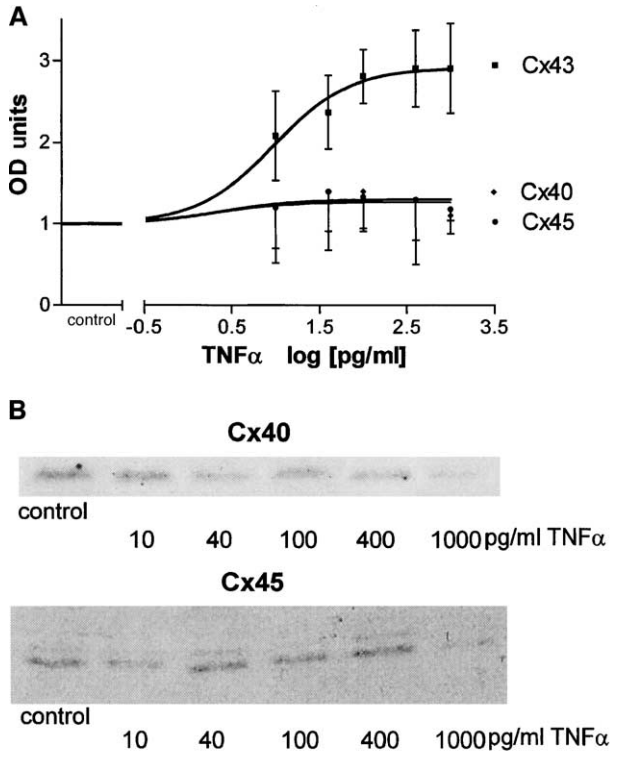

Fig. 2. (A) Dose–response curve: Western blot results for connexin43, connexin40, and connexin45 in neonatal rat ventricular cardiomyocytes exposed for 24 h to increasing concentrations of TNFα (10, 40, 100, 400, and 1000 pg/ml). All values are given as means±S.E.M. of n=5 experiments in optical density (OD) units relative to untreated control cells (see Methods). (B) Original Western blots for connexin40 and connexin45 in neonatal ventricular cardiomyocytes exposed for 24 h to increasing concentrations of TNFα (10, 40, 100, 400, and 1000 pg/ml).

influence of 100 pg/ml, TNFα. This increase could be completely suppressed by the p38 MAP kinase inhibitor SB203580 (10 μM). In contrast to connexin43, the connexin40 mRNA expression remained unchanged after stimulation with TNFα with or without SB203580 (Fig. 4).

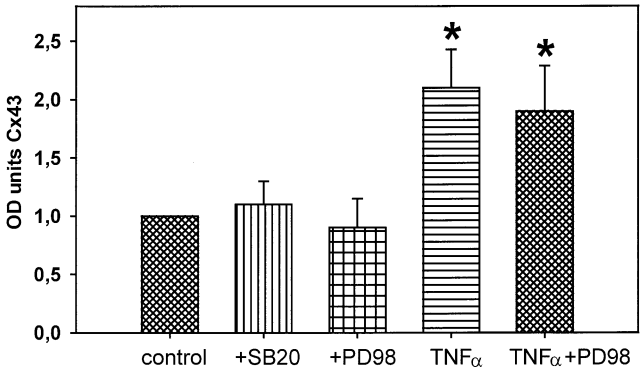

Fig. 3. Western blot results for connexin43 in neonatal rat cardiomyocytes exposed for 24 h to 100 pg/ml TNFα in absence or presence of the p38 MAP kinase inhibitor SB203580 (SB20) or the MEK1 inhibitor PD98059 (PD98). Both inhibitors exerted no effect on Cx43 content in absence of TNFα. All values are given as means±S.E.M. of n=3 experiments. Significant differences vs. control are indicated by an asterisk (P<0.05).

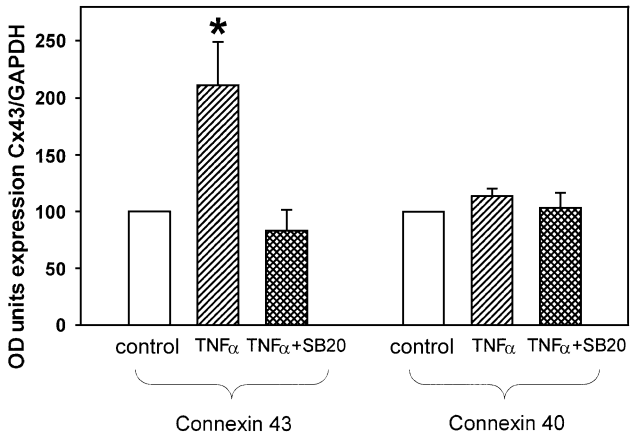

Fig. 4. PCR results for connexin43 mRNA and connexin40 mRNA neonatal rat ventricular cardiomyocytes exposed for 24 h to 100 pg/ml TNFα in absence or presence of the p38 MAP kinase inhibitor SB203580. All values are given as means±S.E.M. of n=5 experiments as relative expression of connexin43 mRNA or connexin40 mRNA related to the housekeeping GAPDH mRNA. Significant differences vs. control (control=100%) are indicated by an asterisk (P<0.05).

3.3. Immunofluorescence staining

The experiments described above were repeated using immunofluorescence histology using a TNFα concentration of 100 pg/ml. In these experiments, enhanced immunostaining for connexin43 was observed in cells exposed to TNFα (Fig. 5). While in control cells not exposed to TNFα connexin43 staining was limited to the intercellular contact zones of the cell membrane, in cells treated with TNFα, we observed enhanced connexin43 immunostaining of the

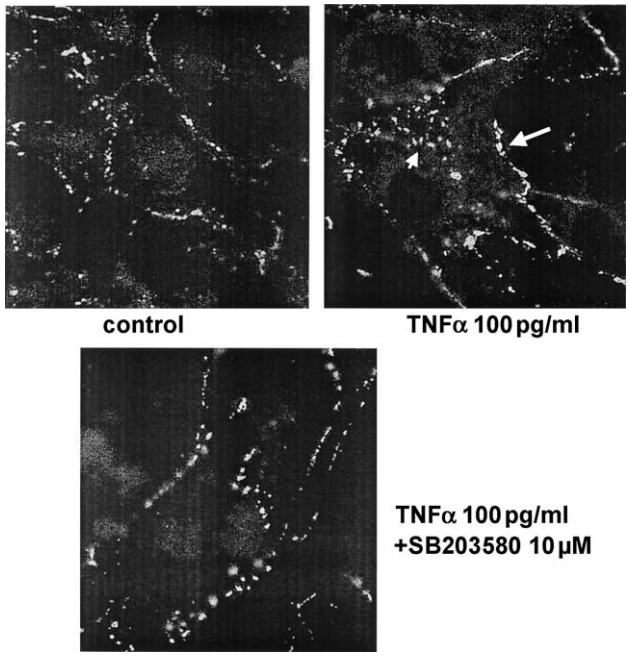

Fig. 5. Immunofluorescent histology staining for connexin43 in neonatal rat ventricular cardiomyocytes with 24-h treatment using 100 pg/ml TNFα with or without the p38 inhibitor SB203580. Note the increased punctuate staining of the cellular border (arrow) and of the cell body (small arrow).

intercellular contact zones of the membrane as well as in the middle of the cell. The TNFα-induced increase in connexin43 immunostaining was completely suppressed in the presence of the p38 MAP kinase inhibitor SB203580 (10 μM).

4. Discussion

Gap junction proteins are essential for intercellular communication, and with respect to the heart, they allow a regular propagation of the electrical impulses. Inasmuch as both expression and distribution of connexins form the basis of the biophysics of the heart tissue and influence the networking, changes in the connexin pattern may lead to cardiac arrhythmia, which are characteristic of chronic heart diseases (Dupont et al., 2001). In our study, we examined the effects of a chronic exposure of the inflammatory cytokine TNFα on cultured neonatal rat cardiomyocytes. Inasmuch as the half-life time of connexin43 is rather short (1.5 h; Darrow et al., 1995, 1996), it can be assumed that, within 6 h (i.e., 4 half-life times), probably the total connexin43 in the membrane is exchanged. Thus, taking this short half-life time into account, 24 h can be considered to resemble a chronic period, and changes in gap junction expression should become evident, although under in vivo conditions, the periods of cardiac disease are considerably longer.

In general, this study demonstrates that the expression of gap junction proteins is not constant but can be regulated, and the results demonstrate a p38 MAP kinase-dependent up-regulation of the connexin43 by activation of a tyrosine kinase-coupled receptor, i.e., the TNFα-receptor. In contrast, the gap junction proteins connexin40 and connexin45, which are known to be found at low levels in the conduction system of the ventricle (Kaprielian et al., 1998), remained constant. However, the specific MEK1-inhibitor PD98059 had no influence on the TNFα-dependent up-regulation of connexin43. This up-regulation seems to involve an increase of connexin43 synthesis, as could be confirmed with the PCR-experiments, showing an increase of connexin43 mRNA. The EC_{50} of the TNFα-effect on connexin43 expression was 9.36±0.48 pg/ml and the C_{max} in the order of 400 pg/ml, which is within the range of TNFα levels which have been observed in septical patients (200–500 pg/ml; Grau et al., 1989; Kwiatkowski et al., 1990; Perkins et al., 2000). Thus, the observed effect may be of pathophysiological relevance (but see below). The sensitivity of the observed increases in connexin43 protein to p38 inhibition by SB203580 indicated a possible regulation on the transcriptional level. It should be noted, however, that according to the literature at 10 μM of SB203580, a partial inhibition of LCK, GSK3, and PKBα can occur (Davies et al., 2000 and see Methods). However, these kinases are not known to be involved in regulation of connexins so far. The view of a regulation on the transcriptional level is further supported by our PCR findings showing enhanced connexin43 mRNA signals also sensitive to SB203580. Thus, we conclude that the changes seen in our study regarding connexin43 resemble changes on the transcriptional level. This enhanced synthesis of connexin43 is mirrored by an enhanced punctuate staining at the cell borders as well as enhanced immunopositivity in the cell body, as revealed by immunohistology. However, effects on the turnover rate or degradation rate cannot be excluded.

Our data show that TNFα-receptor stimulation induced an increase in connexin43, which could not be antagonised by the MEK-1 inhibitor PD98059 but was completely suppressed by SB203580, indicating that TNFα also led to p38 MAP kinase activation and p38 MAP kinase-dependent (and not MEK-1-dependent) regulation of connexin43. This is in good accordance to the literature which demonstrated that TNFα transduces its effects via TNFα-receptor-associated factors (TRAF1-6) and simultaneous activation of NF-κB (nuclear factor-κB), JNK (c-*Jun* N-terminal kinase), and p38 MAP kinase (Eder, 1997). However, regarding the TNFα-effect on connexin expression, there are diverging results reported in the literature: in lipopolysaccharide (LPS)-induced cardiac inflammation in rat hearts, Fernandez-Cobo et al. (1999) found a down-regulation of connexin43 mRNA, following the application of bacterial lipopolysaccharide in an in vivo rat model, while on the other hand, in lung and kidney, connexin43 was increased following lipopolysaccharide exposure (Fernandez-Cobo et al., 1998) as was also found in liver (González et al., 2002). In transfected H92c cells, connexin43 promotor activity was reduced by TNFα (2–500 ng/ml; Fernandez-Cobo et al., 1999). In endothelial cells, 0.5 nM TNFα did not influence connexin43 expression but led to down-regulation of connexin40 and connexin37 (Van Rijen et al., 1998). In contrast, TNFα-induced increases in connexin43 were found in microglia cells, provided that TNFα was applied in the presence of interferon-gamma while it did not influence connexin43 if applied alone (Eugenin et al., 2001). Thus, in different cell types, obviously different signalling pathways connected to connexin43 regulation are activated in response to TNFα-receptor stimulation. Furthermore, regarding the work of Fernandez-Cobo et al. (1999), it has to be taken into account that lipopolysaccharide (LPS) induces a septic shock involving many other factors and complex hemodynamic changes, while we have investigated solely TNFα. In support of our findings, McLaughlin et al. (1996) have shown that TNFα can stimulate MAPK-activated protein kinase 3 (MAPKAP kinase 3), which is a substrate of p38 MAP kinase. In addition, Klein et al. (2002) showed that TNFα can cause phosphorylation of p38 MAP kinase. Thus, if in our model (neonatal rat cardiomyocytes) TNFα increases connexin43 via p38 MAP kinase, and in the model of Fernandez-Cobo et al. (1999) (transfected H9c2 cells) TNFα down-regulates connexin43, it might be speculated that these might be due to additional activation of negative regulators in the H9c2

cells. This further supports our above annotation that there might be cell specificity in the signalling pathways regulating connexin43.

The observed increase in connexin43 expression after TNFα-exposure may be of pathophysiological relevance, inasmuch as it occurs at concentrations which are reached in septic patients (see above). While the recent clinical trials (RENEWAL and ATTACH; Mann et al., 2004; Chung et al., 2003) are against a predominant role of TNFα in heart failure, the data of our study may open the possibility that TNFα may be involved in the remodelling process of the gap junction architecture as a contributing factor. However, to be of functional relevance, the newly synthesised connexin43 has to be integrated into the membrane and has to form functional channels with the neighbouring cells. Immunohistology indicated enhanced punctuate staining at the cell borders. However, it remains to be elucidated in further double cell patch clamp studies whether the increased presence of connexin43 is accompanied by enhanced coupling. Moreover, for arrhythmogenesis, the localisation with respect to the cell poles of the newly formed connexin43 and possible inhomogeneity is of importance. Thus, TNFα-induced differential regulation of connexin43 (and not connexin40 or connexin45) might contribute to arrhythmogenesis (e.g., in septic patients). However, further studies should consider whether in these patients connexin43 is distributed regularly (i.e., at the cell poles) or irregularly (i.e., at cell poles and lateral borders; thus altering anisotropic properties), and whether the enhanced connexin43 presence is associated with enhanced intercellular coupling.

Taken together, our results are in support of the hypothesis that, in neonatal rat cardiomyocytes, TNFα differentially regulates connexin expression and leads to increased connexin43 expression by activation of p38 MAP kinase. This may open possibilities for new pharmacological strategies for control of connexin content.

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