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## **Editorial: Myocardial Ischemia and the Immune System: Some Thoughts and Changing Views**

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## Immunological Implications in Experimental Myocardial Ischaemia: MPO (Myeloperoxidase) Expression Is Differentially Regulated by Beta-Blockers, While CD80 Expression Remains Unaffected

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*In this article, we present some examples of differential gene expression between nebivolol and atenolol. However, from these data we can certainly deduce that beta-blockers unfold a large number of molecular actions. This is reflected in a particular molecular signature, a differential gene expression both in well-oxygenated and ischaemic preparations.*

*In earlier publications, we identified a specific molecular signature of myocardial ischaemia, which possibly equals the severity and type of tissue damage produced – on the other hand, it may demonstrate the activation of repair mechanisms and changes in the metabolic state of the cell.*

*In the presence and absence of beta-blockers, we have seen that numerous intracellular pathways and processes during ischaemia are affected, which are related to ischaemia and cardio-protection. Using PCR for validation, we find that, during experimental ischaemia, there is an up-regulation of MPO expression. There is a differential regulation between different beta-blockers during myocardial ischaemia, which warrants further investigation.*

*We believe that there are complex pleiotropic effects of beta-blockers on T-cell immunity. Such pleiotropic effects have recently received more attention. For example, in the JUPITER trial, in apparently healthy persons without hyperlipidaemia but with elevated high-sensitivity C-reactive protein levels, rosuvastatin significantly reduced the incidence of major cardiovascular events by unfolding pleiotropic anti-inflammatory actions. Our preliminary results show that beta-blockers inhibit the expression of T-cell immunity-related genes during experimental hypoxia. However, a further detailed exploration on both expression and molecular levels is certainly needed. Using PCR, we also tested for CD80: it can be seen that during experimental ischaemia, there is an up-regulation of CD80 expression, however, not statistically significant. There is also a regulation with and without the influence of beta-blockers during myocardial ischaemia.*

*The main message we believe to have taken so far from our investigations is that the most important, unique pleiotropic effects of beta-blockers may be centred around favourable effects upon the immunological response to ischaemia as well as around cardio-protection. Future clinical studies shall investigate the specific immunological significance of these molecular pathways within the framework of cardio-protection. J Clin Basic Cardiol 2011; 14: 16–22.*

**Key words:** CD80, myocardial ischaemia, myeloperoxidase

Ischaemic heart disease is still the main cause of death in the Western hemisphere despite considerable improvement of primary and secondary prevention, diagnostic techniques, and treatment over the past years. Lack of understanding of molecular mechanisms of anti-ischaemic cardio-protection and dysfunctional metabolism contribute largely to cardiovascular disease morbidity and mortality. Ample epidemiologic and clinical evidence suggests that cardiovascular disease will remain the number-one threat to human health in the decades to come. In this context, comparatively little is known about molecular mechanisms in CHD and myocardial tolerance towards ischaemia. However, the topic has recently received considerable attention. The investigation of multiple gene expression patterns during ischaemia, hence, appears to be of high priority in fighting coronary death [1].

However, little is known about the molecular effects of nebivolol upon myocardial ischaemia. In an earlier work, we identified the specific molecular signature of myocardial ischaemia, which, on the one hand, represents the severity and

type of tissue damage produced [2]. On the other hand, it demonstrates the activation of repair mechanisms and changes in the metabolic state of the cell. Doing so in the presence and absence of beta-blockers now allows for conclusions about in which direction cardio-protective mechanisms of these drugs should be further investigated [3].

While it has been shown by a number of authors that in experimental/clinical myocardial ischaemia gene expression is altered, gene expression profiling in ischaemia has not been looked at in human myocardial tissue in depth so far. In earlier publications, we showed that ischaemia can be related to altered patterns of gene expression. The latter, in form of a particular molecular signature, reveals a new understanding of cardio-protective mechanisms. Using this technique, relevant cardio-protective properties of various anti-ischaemic drugs could be more easily assessed. In order to gain more understanding of the relevant molecular pathways and biochemical processes, the effect of beta-blockers has been looked at in this project [4–8].

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## Hypotheses

1. Ischaemia in human myocardial tissue induces an increase in gene expression of particular pathways and processes.
2. Gene expression of other certain pathways and biological processes is decreased in simulated ischaemia.
3. Beta-blockers, known for their anti-ischaemic properties, affect certain pathways and biological processes during simulated ischaemia; these can be identified.
4. Different biochemical variants of beta-blockers entail different anti-ischaemic actions which can be identified at the level of gene expression.
5. These effects can be seen in specific immunological responses.

## Long-Term Expectations

From our experiments, we have learned about myocardial gene expression both in ischaemic heart disease and under cardio-protective therapy. In the long run, our results may provide new approaches in primary prevention as well as in direct myocardial tissue protection during ischaemic heart disease.

We looked at the effects of ischaemia on mRNA expression in experimentally ischaemic human myocardial tissue. In human tissue, myocardial ischaemia leads to an altered molecular signature as we have published before. Results from molecular profiling during myocardial ischaemia indicate an involvement in anti-ischaemic protection and appear to be a major controlling site of metabolism and cardio-protection in low-flow ischaemia. While many experimental studies suggest that changes in gene expression within the ischaemic myocardium help to protect myocardial cells from irreversible injury, little is known in this context about human cardiac ischaemic gene expression and the interrelation between the latter and cardio-protective agents.

Experimental evidence suggests a crucial role of T-lymphocytes in the pathophysiology of atherosclerosis and acute myocardial infarction (AMI). From the bottom of an imaginary inverted pyramid, a few regulatory T-cells control the upper parts represented by the wide spectrum of the inflammatory cascade. In AMI, a pleiotropic pro-inflammatory imbalance with damaging effects in terms of left ventricular performance and patient outcome is the result of this uncontrolled T-cell immune response [9].

In the presence and absence of beta-blockers, we have seen that numerous intracellular pathways and processes during ischaemia are affected, which are related to ischaemia and cardio-protection. Using PCR for validation, we find that, during experimental ischaemia, there is an up-regulation of MPO expression. There is a differential regulation between different beta-blockers during myocardial ischaemia, which warrants further investigation.

We believe that there are complex pleiotropic effects of beta-blockers on T-cell immunity. Such pleiotropic effects have recently received more attention. For example, in the JUPITER trial, in apparently healthy persons without hyperlipidaemia but with elevated high-sensitivity C-reactive protein levels, rosuvastatin significantly reduced the incidence of major cardiovascular events by unfolding pleiotropic anti-inflammatory actions [10]. Our preliminary results show that beta-blockers inhibit the expression of T-cell immunity-related genes during experimental hypoxia. However, a further detailed exploration on both expression and molecular levels is certainly needed. Using PCR, we also tested for CD80: it can be seen that during experimental ischaemia, there is an up-regulation of CD80 expression, however, not statistically significant. There is also a regulation with and

without the influence of beta-blockers during myocardial ischaemia.

The main message we believe to have taken so far from our investigations is that the most important, unique pleiotropic effects of beta-blockers may be centred around favourable effects upon immunological response to ischaemia as well as around cardio-protection. Future clinical studies shall investigate the specific immunological significance of these molecular pathways within the framework of cardio-protection.

## Methods

We looked at the effects of ischaemia on mRNA expression in experimentally ischaemic human myocardial tissue. Myocardial ischaemia, in human tissue, leads to an altered molecular signature, as we published earlier [2]. Results from molecular profiling during myocardial ischaemia taken from preliminary experiments performed at the ZMF (Centre of Medical Research, Medical University Graz) indicate an involvement of beta-blockers in anti-ischaemic protection and appear to constitute a major controlling site of metabolism and cardio-protection in low-flow ischaemia [11]. The present project is designed to study multiple gene expressions involved in myocardial metabolism under ischaemic conditions using an appropriate experimental chamber (see methods). The Panther software was used to obtain specific understanding of possible pathways involved in response to ischaemia and these shall be verified by real-time PCR (Light Cycler). We wished to see whether or not there is a difference in gene expression of metabolic pathways and processes in patients with and without beta-blockade under ischaemic conditions. Doing so, we used human myocardial tissue from the right auricle of patients undergoing cardiac surgery. We studied 4 + 1 subjects in each group. Details of methods and a time table of experimental work can be seen below. Results were statistically evaluated in co-operation with the "Biostatistische Beratung ZMF" and the Institute of Medical Statistics and Biostatistics of the Medical University Graz. We expect to learn from these results whether or not ischaemic myocardial tissue from patients with and without beta-blockade shows altered patterns of metabolic gene expression profiles in order to improve our understanding of anti-ischaemic protection. In particular, we looked at the expression of MPO (myelo-peroxydase) and CD80. In this context, 2 different beta-blockers were tested. Experimental techniques of ischaemia have been well-established by our group over the past 25 years [3] and gene expression measurements [12] have been equally established during our co-operation with Core Facilities Molecular Biology, ZMF, for several years and are constantly in use. There is a good experience of all co-workers involved in the running project.

1. Myocardial tissue probes will derive from the right auricle of patients undergoing cardiac surgery. A small part of the right auricle is removed when the heart is put on extracorporeal circulation and is normally wasted. The muscle piece will then be placed in cooled tyrode solution and transported to the laboratory where it shall be placed into the experimental chamber, as has been done in earlier experiments. The preparation will be oxygenated and then snap-frozen. The model allows for chemically and mechanically induced hypoxia/ischaemia by switching oxygen to nitrogen (hypoxia). Then, real-time PCR (Light Cycler) will be used, based on the works of Schmittgen et al [13], Depra et al [14], Livak et al [15], and our own technical experience. The combined technique of working on a living human preparation in an experimental

chamber and the application of real-time PCR with a Light Cycler has been established in our laboratory of experimental cardiology over a period of several years in order to be able to answer the particular question of the interactions of hypertension, ischaemia, and gene expression in the human heart. Here, the myocardial strip is instantly placed in well-oxygenated, cooled tyrode solution and transported to the laboratory where it is fixed in an experimental chamber. The preparation then is oxygenated (100 % O<sub>2</sub>) before it is cut in 2 pieces of about equal size. Each of the preparations is then put into another individual chamber, as has been done in earlier experiments. Experimental ischaemia is brought about by switching 100 % oxygen to 100 % nitrogen (hypoxia) in one of the chambers [16]. After 30 minutes of simulated ischaemia the tissue probes are snap-frozen using liquid nitrogen. By doing so, we are able to compare ischaemic and non-ischaemic tissues of the same patient. Snap frozen samples are stored at -170 °C until RNA isolation. Preparation for RNA isolation is made by homogenisation of the paraffin-embedded probes using a cryostatic microtome (HM 560 CryoStar; Microm).

2. Solutions: The preparations are continuously perfused with tyrode solution containing (in mM): NaCl 140, KCl 4.5, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.0, glucose 10, and HEPES 20. Solutions were adjusted to a pH of 7.4 by titration with 4 M NaOH and equilibrated with 100 % O<sub>2</sub> at 37 °C [17]. Nebivolol experiments were performed by using a 0.002269 molar stem solution (0.23 g nebivolol + 250 ml aqua dest) whereof 0.5 ml were added to 50 ml tyrode, which results in a 22.47 micromolar solution.
3. RNA isolation and cDNA transcription [13, 14]: total RNA is extracted using the Trizol<sup>®</sup> method (Invitrogen Corp, Carlsbad, CA, USA) and further purified using the RNeasy Mini Kit (Qiagen Inc, Hilden, Germany). After drying, the pellet containing isolated RNA is re-suspended in approximately 30 µl TE buffer. Then quality as well as quantity of RNA are assessed using spectro-photometry. The quality of isolated RNA is also analysed by means of the Agilent's Bioanalyzer 2100 system. Either 20–40 µg or 0.2–1 µg total RNA, when only reduced amounts of material are available, are then directly or indirectly, via in-vitro transcription, transcribed into DIG-labelled cDNA. For reverse transcription of isolated RNA we use the High Capacity cDNA Archive Kit (Applied Biosystems) and the Thermocycler MyCycler<sup>™</sup> from Biorad. Real-time PCR shall be performed using the LightCycler<sup>®</sup> 2.0 System (Roche). Expression of genes is detected using the Taqman format and is mostly compared to the house-keeping gene glucose 6 phosphate dehydrogenase, which is measured using the hybridisation probe format with a kit from Roche (LightCycler – h – G6PDH Housekeeping Gene Set). We then use Taq DNA polymerase for mastermix in both (LightCycler<sup>®</sup> DNA Master HybProbe). Forward primer (= primer 1), reverse primer (= primer 2), as well as the specific complementary Taqman probe are produced by TIB MolBiol Company. Then the expression ratio is calculated.
4. Microarray [12]: The labelled probes are hybridised onto the array for 16 hrs. Subsequently, arrays are washed and detection is carried out using alkaline-phosphatase-conjugated anti-DIG antibodies and the appropriate substrate according to a highly standardized protocol. Arrays are scanned with the AB1700 Chemiluminescence Array Reader and images, raw data, and tissue information are stored in an MIAME-compliant ORACLE dat AB1700 Microarray Analyzer System: The full-genome Chemiluminescence Microarray System (Applied Biosystems) im-

plemented at the Molecular Biology Core Facility of the Center for Medical Research combines the most comprehensive gene probe set with a chemiluminescence-based detection system that is superior to the commonly used fluorescent detection systems with respect to sensitivity (femtomolar level). Expression levels of up to 54,000 transcripts can be measured in one single experiment. About 74 % of the immobilized probes are CELERA-curated (CDS) sequences from public databases and 25 % of the sequences are only accessible via CDS. About 4700 control spots are used to assess array performance in each single step from array fabrication to assay read-out. Another unique feature of the AB1700 system is the co-immobilization of an artificial oligonucleotide probe together with the gene-specific probe in each spot. This probe is detected via a fluorescently labelled oligo-nucleotide that is added to the hybridisation mixture. This novel approach is crucial for optimal localization of each spot and allows normalization strategies that do not necessarily depend on a high-end bio-informatics support. In each experiment, we use pools data of 4 experiments as well as one individual single experiment (n = 4 + 1). The Panther software has been used to process the results statistically and according to biological pathways and biochemical processes [18].

5. Operator(s): The AB1700 microarray system available at the Core Facility Molecular Biology is operated by a team consisting of 4 members with many years of experience in various microarray technologies and platforms (Affymetrix, cDNA- and Oligonucleotide Arrays) [19]. To evaluate the performance of our Applied Biosystems microarray technology, a multicentre proof-of-principle study was conducted by the CF-MB, which included Affymetrix, cDNA- and oligonucleotide-based platforms and that was performed at approved international facilities. The novel AB1700 chemo-luminescence microarray system turned out to be superior with respect to sensitivity and reliability.
6. Ethics committee: The study is in accordance with good clinical and scientific practice. It has been approved by the local ethics committee. All patients undergoing cardiac surgery gave informed consent that the small part of their right auricle, which would have normally been wasted, could be used for scientific investigation for this specific study.

## Results

### MPO (Myeloperoxidase) Expression Is Up-Regulated in Simulated Myocardial Ischaemia in the Presence of Nebivolol

Experimental evidence suggests a crucial role of T-lymphocytes in the pathophysiology of atherosclerosis and acute myocardial infarction (AMI). From the bottom of an imaginary inverted pyramid, a few regulatory T-cells control the upper parts represented by the wide spectrum of the inflammatory cascade. In AMI, a pleiotropic pro-inflammatory imbalance with damaging effects in terms of left ventricular performance and patient outcome is the result of this uncontrolled T-cell immune response [9]. Our results show that nebivolol, not atenolol, inhibits activation of T-cell immunity either secondarily by effective cardio-protection or by directly modulating T-cell immunity [19, 20].

Experimental and clinical evidence also suggests that hypoxic/ischaemic damage induces angiogenesis within a very short time. The cellular injury that results from irreversible ischaemia leads to left ventricular re-modelling. Oxidative stress and inflammation are key elements of this process; el-

evaluation of markers of inflammation at the time of myocardial infarction is a predictor of the development of ischaemic cardiomyopathy and of adverse clinical outcomes. Several leukocyte-derived enzyme systems are responsible for the release of oxidizing agents into the myocardium after ischaemic injury and provide a means of better preventing ischaemic damage. For example, such a key leukocyte-derived marker which correlates with outcomes, is myeloperoxidase (MPO) [21, 22].

In our present work, using microarray technique, we have found that, in T-cell-mediated immunity generally, a noteworthy down-regulation is brought about by nebivolol but not by atenolol. In particular, the following genes were inhibited by nebivolol, not atenolol of T-cell immunity-related genes during experimental hypoxia: GRB2-ASSOCIATED BINDER-1, GAB1 (PTHR12156:SF3), TYROSINE-PROTEIN KINASE TEC/TXK (PTHR23256:SF263), MHC CLASS II ALPHA CHAIN (PTHR19944:SF27), T CELL SURFACE GLYCOPROTEIN CD6 (PTHR19331:SF12), TAPASIN (PTHR23411), MHC CLASS II ALPHA CHAIN (PTHR19944:SF27), ELF2 (PTHR11849:SF10), SCHLAFEN 5 (PTHR12155:SF5), SCHLAFEN RELATED (PTHR12155:SF6), T CELL CO-STIMULATION ANTIGEN CD80 (B7-1) (PTHR13712:SF44), MHC CLASS II ALPHA CHAIN (PTHR19944:SF27), T-CELL SURFACE GLYCOPROTEIN CD3 EPSILON CHAIN (PTHR10506), T-CELL SURFACE GLYCOPROTEIN CD3 EPSILON CHAIN (PTHR10506), T-CELL RECEPTOR BETA CHAIN V REGION LB2 (PTHR23268:SF7), T-CELL RECEPTOR BETA CHAIN V REGION C5 (PTHR23268:SF6), T-CELL RECEPTOR ALPHA CHAIN V REGION-RELATED (PTHR19433), T-CELL RECEPTOR BETA CHAIN V REGION (PTHR23268:SF4), SCHLAFEN RELATED (PTHR12155:SF6), TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY, MEMBER 25 (PTHR23097:SF38) [19, 20].

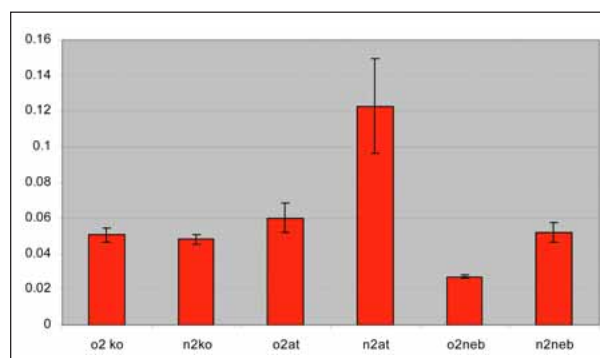
From our investigations we suspect that the most important, unique pleiotropic effects of nebivolol may be centred around favourable effects upon a T-cell-mediated response to ischaemia [9]. In our microarray experiments, we found an up-regulation of MPO expression in the presence of nebivolol as well as in the presence of atenolol both in hypoxic and in well-oxygenated conditions, as can be seen in Table 1. Using PCR for validation, we find that during experimental ischaemia, there is an up-regulation of MPO expression (Figure 1). There is a differential regulation between different beta-blockers during myocardial ischaemia, which warrants further investigation.

We believe that there are complex pleiotropic effects of beta-blockers on T-cell immunity. Such pleiotropic effects have recently received more attention. For example, in the JUPITER trial, in apparently healthy persons without hyperlipidaemia but with elevated high-sensitivity C-reactive protein levels, rosuvastatin significantly reduced the incidence of major cardiovascular events by unfolding pleiotropic anti-inflammatory actions [23]. Our preliminary results show that beta-blockers inhibit the expression of T-cell immunity-related genes during experimental hypoxia. In the light of JUPITER and other recent publications on modulating inflammation by pleiotropic effects of cardiovascular drugs, the specific property of T-cell modulation by nebivolol in myocardial ischaemia may warrant further attention. However, a further detailed exploration on both expression and molecular levels is certainly needed.

Nebivolol reduced the expression of pro-inflammatory genes in endothelial cells and vascular smooth muscle cells in vitro, whereas metoprolol did not. In vivo, nebivolol inhibited neointima formation by reducing SMC proliferation and

**Table 1.** MPO is 1.3× up-regulated in nebivolol under hypoxia and 4× under normoxia. MPO is 350× up-regulated in atenolol under hypoxia and 1.5× under normoxia.

	N <sub>2</sub> hypoxia	
	Nebivolol (control)	Atenolol (control)
MPO	1.34	350.42
	O <sub>2</sub> normoxia	
	Nebivolol (control)	Atenolol (control)
MPO	4.73	1.57



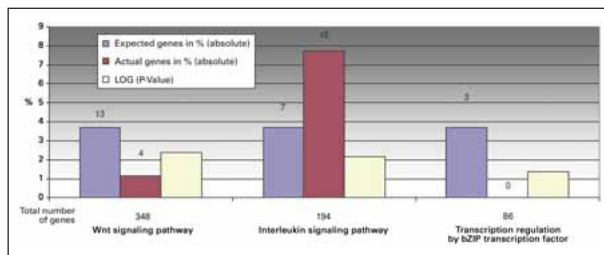
**Figure 1.** It shows the results from real-time PCR measurements of all MPO experiments (o2ko: well-oxygenated, no ischaemia, no drug; n2ko: experimental ischaemia, no drug; o2at: well-oxygenated, no ischaemia, atenolol present; n2at: experimental ischaemia, atenolol present; o2neb: well-oxygenated, nebivolol present; n2neb: experimental ischaemia, nebivolol present). Using PCR for validation, we find that during experimental ischaemia, there is an up-regulation of MPO expression. There is a differential regulation between different beta-blockers during myocardial ischaemia, which warrants further investigation.

macrophage accumulation [24]. However, a more complex, likely favourable regulation has also been seen in several aspects of early inflammatory pathways.

#### CD80 Antigen Gene Expression Is Not Affected by Either Nebivolol or Atenolol

Neuroendocrine/inflammatory and endothelial functions have been indicated as crucial for heart failure (HF) patients. The effects of carvedilol on cytokines and asymmetric dimethylarginine (ADMA) and left ventricular ejection fraction (LVEF) at baseline and after long-term administration of carvedilol have been investigated by others. Carvedilol appears to reduce symptoms and the expression of inflammation, regardless of LV functional response [25] and nebivolol is as effective as carvedilol in patients with symptomatic chronic heart failure and reduced LV systolic function [26]. Carvedilol is attenuating inflammation, oxidative response, myocardial fibrosis, and apoptosis, as well as in preserving energy transcription factors and LV function in DCM [27]. Nebivolol has not been the first beta-blocker to be attributed an anti-inflammatory potential.

Cardiac injury activates innate immune mechanisms initiating an inflammatory reaction. Immunological receptor-mediated pathways, the complement cascade, and reactive oxygen generation induce nuclear factor (NF) κB activation and up-regulate chemokine and cytokine synthesis in the infarcted heart. Chemokines stimulate the chemo-tactic recruitment of inflammatory leukocytes into the infarct, while cytokines promote adhesive interactions between leukocytes and endothelial cells. These enhance early transmigration of



**Figure 2.** Interleukin pathway up-regulated by nebivolol, not by atenolol, during experimental ischaemia.

**Table 2.** CD80 is more than 3× down-regulated in nebivolol under hypoxia, but 3.9× up-regulated under normoxia. CD80 is up-regulated in atenolol under hypoxia as well as in normoxia.

N <sub>2</sub> hypoxia		
	Nebivolol (control)	Atenolol (control)
CD80	0.23	1.18
O <sub>2</sub> normoxia		
	Nebivolol (control)	Atenolol (control)
CD80	3.92	1.83

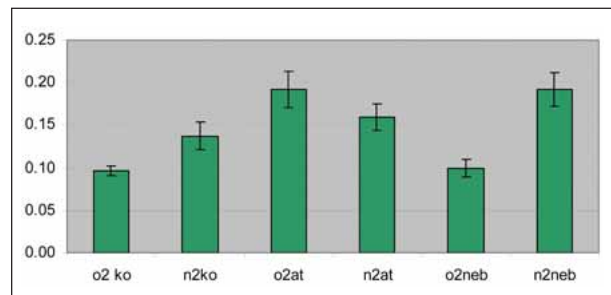
inflammatory cells into the site of injury and, during the early phase of ischaemia, additional cell damage [28].

Higher levels of anti-inflammatory cytokine interleukin-10 provide protection in unstable angina [29]. Interleukin-10 improves LV function in rats with heart failure subsequent to myocardial infarction [30]. We specifically looked at CD80 expression. Using microarray, we found that interleukin pathways are more than 2× up-regulated by nebivolol (Figure 2). CD80, in contrast, is more than 3× down-regulated in nebivolol under hypoxia, but 3.9× up-regulated under normoxia (Table 2). CD80 is up-regulated in atenolol under hypoxia as well as in normoxia.

Inflammatory cytokines are cardio-depressant mainly due to impairment of intracellular Ca(2<sup>+</sup>) homeostasis, leading to decreased contractility and hence locally to a decreased myocardial O<sub>2</sub> demand in the ischaemic area. Inflammatory cytokines stimulate apoptosis through a TNF-α receptor/caspase pathway, whereas Ca(2<sup>+</sup>) overload induced by extensive ROS generation causes necrosis through enhanced permeability of the mitochondrial membrane (mitochondrial permeability transition) [31].

Using PCR, we tested for CD80 (Figure 3); it can be seen that during experimental ischaemia, there is an up-regulation of CD80 expression, however, not statistically significant. There is also a regulation with and without the influence of beta-blockers during myocardial ischaemia.

However, cell-mediated immunity is largely involved in ischaemic damage. In well-oxygenated preparations, we have shown that numerous biochemical processes, mainly those involved in signalling and cellular immunity, are effected by nebivolol but not by atenolol, as can be seen from our more complex microarray data. Here, a more detailed approach is definitely needed. These observations warrant further attention. This shows that, in non-ischaemic, well-perfused preparations, nebivolol unfolds particular, possibly cardio-protective effects which cannot be observed under atenolol. Similarly, we can see that many processes involved in contraction, lipid metabolism, and proliferation are down-regulated by nebivolol only (but not by atenolol). In well-oxygenated preparations, we can see that numerous biological path-



**Figure 3.** It shows the results from real-time PCR measurements of all CD80 experiments (o2ko: well-oxygenated, no ischaemia, no drug; n2ko: experimental ischaemia, no drug; o2at: well-oxygenated, no ischaemia, atenolol present; n2at: experimental ischaemia, atenolol present; o2neb: well-oxygenated, nebivolol present; n2neb: experimental ischaemia, nebivolol present). It can be seen that during experimental ischaemia, there is an up-regulation of CD80 expression. There is also a regulation with and without the influence of beta-blockers during myocardial ischaemia.

ways, mainly those involved in signalling, angiogenesis, cellular immunity, and EGF, are affected by nebivolol but not by atenolol. Concerning nebivolol-regulated biological pathways, we could see that, during normoxia, up-regulation of pathways is not very different between nebivolol and atenolol.

## Discussion

While drugs exert specific class effects within one particular class of drug, each class member may entail additional specific properties. This effect has been called “dirty drug effect” in the past. However, more recently, these effects have increased the interest of scientists. An ideal example is the non-lipid-related effect of statins, which has led to a far more extended application. The complexity of the inflammatory cascade involved in the development of atherosclerosis makes it difficult to develop single-target drugs in order to slow or affect the process. Drugs have always been in search for new indications and atherosclerosis has been a target ever since. Just very recently, it has been shown that inflammation *per se* could be an attractive target for pharmacological intervention in primary prevention. The JUPITER trial has directly addressed this problem: in apparently healthy persons without hyperlipidaemia but with elevated high-sensitivity C-reactive protein levels, a statin significantly reduced the incidence of major cardiovascular events by unfolding pleiotropic anti-inflammatory actions [10, 23]. Similar effects have recently been discussed for beta-blockers as well. The latter constitute a rather heterogeneous group of drugs. It is generally accepted that not protection of endothelial function alone, but a plethora of anti-inflammatory and anti-proliferative actions is brought about by various drugs effective in primary and secondary prevention [32]. More modern techniques, like, eg, microarray expression profiling, have revealed a very complex molecular action of specific drugs. Even within the same class of drugs, the effect upon gene expression profiles varies markedly. Here we may illustrate the point by a brief look at the effect of beta-blockers upon inflammatory mechanisms. Several leukocyte-derived enzyme systems are responsible for the release of oxidizing agents into the myocardium after ischaemic injury and they provide a means of better preventing ischaemic damage. For example, such a key leukocyte-derived marker, myeloperoxidase (MPO), correlates with outcomes [33]. Recent experimental evidence suggests a crucial role of T-lymphocytes in the pathophysiology of atherosclerosis and acute coronary syndromes. It has been indicated that a pro-inflammatory imbalance resulting from

T-cell activation could be responsible for activating the inflammatory cascade ultimately responsible for cellular injury, left ventricular dysfunction, remodelling, and outcome [9]. For example, differential effects on T-cell immunity are seen between different beta-blockers: nebivolol, but not atenolol, inhibits the expression of T-cell immunity-related genes during experimental hypoxia [19, 20]. Certain beta-blockers also reduced the expression of pro-inflammatory genes in endothelial cells and vascular smooth muscle cells *in vitro*, whereas others did not. *In vivo*, beta-blockers inhibited neointima formation by reducing SMC proliferation and macrophage accumulation [34]. Cellular injury that results from irreversible ischaemia leads to LV remodelling. Oxidative stress and inflammation are key elements of this process; elevation of markers of inflammation at the time of myocardial infarction is a predictor of the development of ischaemic myopathy and of adverse clinical outcomes.

Several leukocyte-derived enzyme systems are responsible for the release of oxidizing agents into the myocardium after ischaemic injury and provide a means of better preventing ischaemic damage. For example, such a key leukocyte-derived marker, myeloperoxidase (MPO), correlates with outcomes.

Proteins of the matrix metalloproteinase (MMP) family are normally involved in the turnover of extracellular matrix, eg, during reproduction, embryonic development, and tissue remodelling. MMPs show a wide field of action also in disease processes, in particular during cardiac hypertrophy and ischaemia. MMPs are also targeted by statins in cardiovascular primary and secondary prevention. A recent paper supplies direct experimental evidence that metoprolol reduces inflammation in vulnerable plaques [35]. However, a more complex, likely favourable regulation has also been seen in several aspects of early inflammatory response: neuroendocrine/inflammatory and endothelial functions have been indicated as crucial not only in the development of atherosclerosis [33] but also for heart failure (HF) patients.

For example, nebivolol constitutes a special type of beta-blocker, which shows properties different from those of other beta-blockers [36, 37]. It exerts various additional effects, such as acting on the endothelial NO pathway [38, 39] and an extremely high affinity to the  $\beta_1$ - and  $\beta_3$ -adrenoceptor. It also shows a high affinity to serotonin receptors of the 5-HT<sub>1A</sub> subtype and has been attributed a considerable cardioprotective effect [40, 41]. During and after myocardial ischaemia, beta-blockers support the preservation of myocardial integrity. Since nebivolol shows numerous pharmacological properties which may entail anti-ischaemic protection, we have analysed the expression of 27,184 genes in human atrial myocardium under ischaemic conditions in the presence and absence of nebivolol and compared it to atenolol. However, little is known about the molecular effects of nebivolol upon myocardial ischaemia [42, 43]. In the present work, we have identified the specific molecular signature of beta-blockers in myocardial ischaemia, which, on the one hand, represents the severity and type of tissue damage produced. On the other hand, it demonstrates the activation of repair mechanisms and changes in the metabolic state of the cell. Doing so in the presence and absence of nebivolol and atenolol will allow for conclusions about in which direction cardioprotective mechanisms of the drug should be further investigated.

Quality as well as quantity of isolated mRNA were double-checked using both spectrophotometry as well as on Agilent's Bioanalyzer 2100 system. The AB1700 microarray system available at the Core Facility Molecular Biology is operated by a team consisting of 4 members with many years of experience in various microarray technologies and platforms

(Affymetrix, cDNA, and oligonucleotide arrays). To evaluate the performance of the novel applied Biosystems microarray technology, a multicentre proof-of-principle study was conducted by the CF-MB, which included Affymetrix, cDNA- and oligonucleotide-based platforms and that was performed at approved international facilities. The novel AB1700 chemiluminescence microarray system turned out to be superior with respect to sensitivity and reliability. Hence, we believe that data are highly reliable.

One can see that, in the presence of nebivolol, processes that are mainly involved in myocardial ischaemia, damage, and inflammation are down-regulated, while processes involved in structural integrity, circulation, gas exchange activity, glucose homeostasis, and other vital cellular mechanisms expressing myocardial anti-ischaemic protection are significantly more expressed. From these data one can conclude that nebivolol exerts an important cardioprotective action on a variety of intracellular pathways.

Experimental evidence suggests a crucial role of T-lymphocytes in the pathophysiology of atherosclerosis and acute myocardial infarction (AMI). From the bottom of an imaginary inverted pyramid, a few regulatory T-cells control the upper parts represented by the wide spectrum of the inflammatory cascade. In AMI, a pleiotropic pro-inflammatory imbalance with damaging effects in terms of left ventricular performance and patient outcome is the result of this uncontrolled T-cell immune response [9, 33]. In the presence and absence of beta-blockers, we have seen that numerous intracellular pathways and processes during ischaemia are affected, which are related to ischaemia and cardio-protection. Using PCR for validation, we find that, during experimental ischaemia, there is an up-regulation of MPO expression. There is a differential regulation between different beta-blockers during myocardial ischaemia which warrants further investigation.

We believe that there are complex pleiotropic effects of beta-blockers on T-cell immunity. Such pleiotropic effects have recently received more attention. Our preliminary results show that beta-blockers inhibit the expression of T-cell immunity-related genes during experimental hypoxia. However, a further detailed exploration on both expression and molecular levels is certainly needed. Using PCR, we also tested for CD80: it can be seen that during experimental ischaemia, there is an up-regulation of CD80 expression, however, not statistically significant. There is also a regulation with and without the influence of beta-blockers during myocardial ischaemia. However, cell-mediated immunity is largely involved in ischaemic damage. In well-oxygenated preparations, we have shown that numerous biochemical processes, mainly those involved in signalling and cellular immunity, are effected by nebivolol but not by atenolol, as can be seen from our more complex microarray data. Here, a more detailed approach is definitely needed. The observations warrant further attention.

Similarly, we can see that many processes involved in contraction, lipid metabolism, and proliferation are down-regulated by nebivolol only (not by atenolol). In well-oxygenated preparations, we can see that numerous biological pathways, mainly those involved in signalling, angiogenesis, cellular immunity, and EGF, are affected by nebivolol but not by atenolol. The effect of carvedilol on cytokines, asymmetric dimethylarginine (ADMA), and left ventricular ejection fraction (LVEF) at baseline and after long-term administration of carvedilol has been studied and carvedilol reduced symptoms as well as parameters of inflammation regardless of the left ventricular functional response [25]. Carvedilol has also been shown to attenuate inflammation, oxidative response, myocardial fibrosis, and apoptosis, as well as to preserve energy

transcription factors and LV function in DCM [44]. It is of ten cardiac injury itself that propagates immune mechanisms leading to an inflammatory reaction [28]: on the one hand, immunological receptor-mediated pathways and the complement cascade lead to the activation of nuclear factor (NF- $\kappa$ B) and increased chemokine and cytokine syntheses in the ischaemic tissue, thus attracting inflammatory leukocytes into the ischaemic area. On the other hand, cytokines promote adhesive interactions between leukocytes and the endothelial layer, enabling transmigration of inflammatory cells into the site of injury. Inflammatory cytokines stimulate apoptosis through a TNF- $\alpha$  receptor/caspase pathway [45]. Inflammatory mechanisms thus play a role in both the development of atherosclerosis and the effect of coronary artery disease.

In summary, there is a complex relationship between beta-adrenergic blockade, sympathetic activity, and inflammation [46]. The next decade will certainly be marked by the investigation of pleiotropic drug effects upon inflammatory processes searching for further and more specific applications of drugs in primary prevention.

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