1. Introduction

1.1 Endothelial inflammatory signaling and monocyte recruitment

Recruitment of circulating monocytes to activated areas of the endothelium and their migration to the subintimal inflammatory foci represents one of the earliest events in atherogenesis (Linton and Fazio 2003; Hansson 2005). Importantly, monocyte recruitment can be recognized throughout all lesional stages including advanced lesions, where plaque infiltration and neovascularization occur. Indeed, available experimental evidence supports the notion that in advanced stages monocyte infiltration contributes to plaque instability and rupture (Virmani, Burke et al. 2006). Monocyte recruitment to the subendothelial milieu implies a sequence of events that begin with monocyte rolling along and tethering to the endothelial surface, firm adhesion and activation, and ultimately migration to the subintima. At the molecular level, the entire sequence entails interaction of integrins on the monocyte surface with cell adhesion molecules (CAMs) expressed on the endothelial cell. Monocyte rolling and tethering is mainly mediated by CAMs from the selectin group (v.g., E-selectin) while firm adhesion and migration are mostly mediated by CAMs from the immunoglobulin (Ig) superfamily, such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Compelling evidence accumulated over the last decade has clearly established that VCAM-1 (CD106) has a prominent role in mediating attachment and migration of monocytes (the Cluster of Differentiation nomenclature (Zola, Swart et al. 2005) is given for reference, but “VCAM-1” will be used throughout the text).

1.1.1 VCAM-1 expression and atherosclerosis

Although other adhesion molecules, such as ICAM-1 (CD54) or E-selectin, also contribute to adhesion of monocytes to endothelial cells, VCAM-1 is unique in that its expression level and pattern are highly sensitive to the action of several pro-inflammatory/pro-atherogenic stimuli. While other CAMs are constitutively expressed in non-activated
endothelium, VCAM-1 is virtually absent, but its markedly upregulated when endothelium is exposed to atherorelevant stimuli (Galkina and Ley 2007). For example, in hypercholesterolemic animals both ICAM-1 and VCAM-1 are induced in early lesions. However, VCAM-1 expression is largely restricted to lesions, or to sites prone to lesion formation, and can also be detected even before the onset of visible fatty streaks, while ICAM-1 also extends into uninvolved aorta and lesion-protected regions (Iiyama, Hajra et al. 1999). This dissimilar pattern immediately suggested different functions for ICAM-1 and VCAM-1, at least in lesion initiation. A more direct comparison of VCAM-1 and ICAM-1 in atherosclerosis was possible through generation of mice homozygous for a VCAM-1 molecule lacking the Ig-like extracellular domain 4 (Vcam1<sup>D4D/D4D</sup>) which partially circumvents the embryonic lethality of Vcam-1<sup>-/-</sup> mice. Using such mouse model it was possible to show that whereas both ICAM-1 and VCAM-1 are upregulated in lesions, VCAM-1 has a dominant role in early lesion formation (Cybulsky, Iiyama et al. 2001; Dansky, Barlow et al. 2001). Atherorelevant stimuli such as tumor necrosis factor-α (TNFα), locally released nucleotides, vascular endothelial growth factor (VEGF) or oxidized low density lipoprotein (oxLDL), are potent inducers of VCAM-1 expression (Galkina and Ley 2007). Depending on the stimulus and/or the regional location of the endothelial cell along the vascular bed, VCAM-1 expression is regulated through Nuclear Factor κappaB (NFκB), Nuclear Factor of Activated T cells (NFAT) or both (Armesilla, Lorenzo et al. 1999; Kim, Moon et al. 2001; Yao and Duh 2004).

1.1.2 VCAM-1 structure and function

Human VCAM-1 is a single transmembrane protein with seven Ig-like extracellular domains (1-7) but can also be expressed as a form with only domains 1-3 and 5-7 (Chuluyan, Osborn et al. 1995). The extracellular domains 1 and 4 mediate specific binding to the integrin α<sub>4</sub>β<sub>1</sub> (Very Late Antigen 4, VLA-4) on the monocyte facilitating firm adhesion to the endothelium. The cytoplasmic domain is only 19 amino acids long, and has a PDZ-binding motif, but specific interactions or functional relevance remain unknown. Interaction of VCAM-1 with α<sub>4</sub>β<sub>1</sub> conveys a conformational message through the transmembrane domain towards the cytosolic region, triggering intracellular signaling events. Importantly, VCAM-1-dependent signaling is a prerequisite for successful migration of bound monocytes (Matheny, Deem et al. 2000; Deem, Abdala-Valencia et al. 2007). Some salient features within this signaling are the recruitment and activation of the Rho-family GTPase Rac-1, Rac-1 dependent stimulation of NADPH oxidase and production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (van Wetering, van den Berk et al. 2003). VCAM-1-dependent release of H<sub>2</sub>O<sub>2</sub> to the extracellular milieu is thought to contribute to activation of matrix metalloproteases and the increased endothelial permeability observed during atherogenesis. This may be particularly important in advanced lesions by creating additional endothelial damage and plaque instability (Virmani, Burke et al. 2006). Calcium (Ca<sup>2+</sup>) release and influx subsequent to VCAM-1-α<sub>4</sub>β<sub>1</sub> interaction or antibody-induced VCAM-1 crosslinking have also been related to the signaling required for monocyte migration (Isabelle Ricard 1997; Cook-Mills 2002; Cook-Mills, Johnson et al. 2004). However, neither the nature of the channels involved nor the underlying mechanism/s are yet known. In coronary artery endothelial cells of human origin (HCAECs) activation of VCAM-1 by antibody-induced crosslinking results in
approximately three-fold increase in the rate of cation influx compared to non-treated cells (Smedlund and Vazquez, unpublished observations). Such increase in cation influx occurs over the existing constitutive influx, and thus is possible that both constitutive and regulated activity of cation channels play a role. Because TRPC3 accounts for most of the constitutive cation entry in HCAECs and also significantly contributes to regulated influx (Vazquez and Putney 2006; Smedlund and Vazquez 2008) it is likely that TRPC3 represents a component of the signaling underlying VCAM-1-dependent monocyte transmigration.

1.2 Role of macrophage survival and apoptosis in lesion development

As mentioned above, monocyte recruitment to the subintima is a fundamental event in atherogenesis (Linton and Fazio 2003). Transmigration of the monocyte to the subendothelial milieu is followed by its differentiation into macrophage, which is now recognized as a key cell component in determining lesion progression and fate. Lesional macrophages engulf modified lipoproteins, mostly oxLDL, becoming lipid-laden macrophages; this results in a lipid overload of the macrophage which imposes a significant stress to the endoplasmic reticulum (ER), mostly due to accumulation of free cholesterol. Consequently, an irreversible ER-stress response is triggered leading to macrophage apoptosis. Indeed, the majority of apoptotic lesional cells are macrophages (Linton and Fazio 2003; Tabas 2010). Clearance of apoptotic cells is conducted by resident phagocytes, which phagocytose the apoptotic macrophage and exit the lesion site through lymphatic circulation or by migrating back to the blood stream. This clearing process, known as efferocytosis, is crucial in preventing the apoptotic cells from dying in situ which would otherwise lead to post-apoptotic necrosis and the subsequent exacerbation of the inflammatory response. Notably, the balance between production of apoptotic macrophages and their clearance by efferocytosis constitutes a defining factor in lesion formation, remodeling and progression (Tabas 2010 and references therein). For instance, macrophage apoptosis in early lesions is beneficial in that reduces lesion cellularity and size and plaque progression (Arai, Shelton et al. 2005; Liu, Thewke et al. 2005; Babaev, Chew et al. 2008; Wang, Liu et al. 2008) while increased apoptosis in advanced plaques enlarges the necrotic core and promotes plaque instability (Linton, Babaev et al. 1999; Seimon, Wang et al. 2009; Yancey, Blakemore et al. 2010). Thus, altered expression and/or deregulation of signaling proteins directly or indirectly involved in macrophage survival/apoptosis can have a significant impact on lesion progression.

2. TRPC channels

2.1 Structure, function and role in cardiovascular disease

Calcium influx has long been recognized as an essential component of physiological and pathophysiological events. Changes in intracellular Ca$^{2+}$ concentration that follow Ca$^{2+}$ influx through plasma membrane Ca$^{2+}$ channels not only modulate a myriad of Ca$^{2+}$-dependent signaling pathways but also affect the driving force for other ions by modifying the membrane potential. Ca$^{2+}$ influx is of particular importance in vascular function and cardiovascular disease, where the effects of Ca$^{2+}$ influx can be seen throughout the entire cardiovascular system, in smooth muscle and endothelial cells, cardiomyocytes,
lymphocytes, monocytes, macrophages, among other cell types. Of the many Ca\(^{2+}\) channels identified in the last half century, Transient Receptor Potential Canonical (TRPC) channels are recognized as major contributors to Ca\(^{2+}\) influx and play a role in various physiological and pathological states. The TRPC family belongs to the TRP superfamily of ion channel forming proteins, and are the most closely related to the founding member Drosophila TRP protein (Vazquez, Wedel et al. 2004). TRPC proteins can be grouped into four subgroups, TRPC1, TRPC2 (a pseudogene in humans), TRPC3/6/7 and TRPC4/5 (Trebak, Vazquez et al. 2003; Vazquez, Wedel et al. 2004).

Despite some structural variation across the subgroups of the TRPC family, there are several structural motifs which are conserved throughout members. The cytoplasmic N- and C-termini are separated by six transmembrane domains (TM1-TM6), with a re-entry loop between TM5 and TM6 which is thought to line the wall of the channel pore ((Vazquez, Wedel et al. 2004) and references therein). Other shared structural motifs of TRPCs include ankyrin repeats and a putative caveolin binding site on the N-terminus, and on the C-terminus the so called TRP signature motif (EWKFAR), a proline-rich motif and a calmodulin/IP\(_3\) receptor binding (CIRB) site; predicted coiled-coil regions are present on both N- and C-termini, and in TRPC4 and 5, an extended C-terminus includes a PDZ binding motif (Vazquez, Wedel et al. 2004). An examination of the function of the TRPC cytoplasmic motifs hints at mechanisms of channel activation and signaling pathways. For instance, ankyrin repeats form specialized structures with the repeated units stacking against one another to form a protein-binding interface; this allows for interaction with other proteins and seems to play a role in channel trafficking to the plasma membrane. Coiled-coil regions are commonly associated with oligomerization and may contribute to formation of specific homo- and heterotetramers of TRPCs or association with other proteins containing coiled-coil motifs (Vazquez, Wedel et al. 2004). The proline-rich motif and CIRB region have also been associated to interactions with different signaling molecules, with variations existing throughout individual TRPC members. The mechanisms underlying activation and regulation of TRPC channels has been matter of extensive research and debate, with efforts mostly centered at elucidating whether they form store-operated (activated by mere depletion of internal Ca\(^{2+}\) stores) or non-store-operated channels (discussed in (Trebak, Vazquez et al. 2003; Vazquez, Wedel et al. 2004; Smyth, DeHaven et al. 2006). Whereas some properties of TRPC channels observed in heterologous expression systems correlate well with those of TRPCs expressed under native conditions, many others do not. It is imperative to elucidate the mechanism/s underlying regulation of TRPC channels in their native environment, as this would greatly contribute to assign definitive roles to individual TRPC members. Nevertheless, equally important to comprehend their role in cardiovascular physiology and disease is to identify cellular and molecular events which, directly or indirectly, may rely upon appropriate TRPC function.

TRPC proteins are ubiquitously expressed throughout the cardiovascular system and hematopoietic cells and all members have been implicated not only in physiological cardiovascular functions but most importantly, in the pathogenesis of cardiovascular disease. Indeed, TRPCs have been implicated in a variety of processes known to be critical in cardiovascular pathology such as endothelial dysfunction, vascular relaxation, oxidative
stress, and angiogenesis among others. This has recently been reviewed by us (Tano, Smedlund et al. 2010) and others (Abramowitz and Birnbaumer 2009) and the reader is referred to those for further details. The following sections focus on recent findings that specifically point to a potential role of TRPC3 in atherorelevant processes.

3. Participation of TRPC3 in atherorelevant molecular/cellular processes

3.1 TRPC3 and regulated expression of VCAM-1

Calcium signaling is an important component of the mechanism by which several inflammatory factors induce VCAM-1 expression. For instance, changes in intracellular Ca\(^{2+}\) associated to Ca\(^{2+}\) release from internal stores have been linked to the ability of Substance P to induce VCAM-1 subsequent to Ca\(^{2+}\)-dependent activation of NFAT and NFκB in microvascular endothelium (Quinlan, Naik et al. 1999), and of β2-microglobulin to induce VCAM-1 expression in synovial fibroblasts (Chen, O'Neill et al. 2002). In the human coronary endothelial cells HCAEC, Ca\(^{2+}\) mobilization contributes to lipoprotein A- and ATP-dependent VCAM-1 expression (Allen, Khan et al. 1998; Seye, Yu et al. 2003). Nevertheless, the specific role of Ca\(^{2+}\) influx in VCAM-1 expression was never directly examined before. In recent work we specifically explored the impact of Ca\(^{2+}\) influx in regulated expression of VCAM-1 in HCAECs (Vazquez and Putney 2006; Smedlund and Vazquez 2008). Our studies demonstrated that, whereas HCAECs express all members of the TRPC family, only TRPC3 forms, or is part of, endogenous Ca\(^{2+}\)-permeable channels that contribute to ATP stimulated Ca\(^{2+}\) influx. Such mechanism occurs downstream ATP-dependent activation of purinergic P2Y\(_2\) receptors and results in upregulation of VCAM-1 total and plasma membrane associated levels with subsequent increase in monocyte adhesion (Smedlund and Vazquez 2008). This represented the first direct indication that Ca\(^{2+}\) influx plays a role in the signaling driving VCAM-1 expression and that TRPC3 forms native Ca\(^{2+}\)-permeable channels whose activity is fundamental within the signaling underlying VCAM-1 expression and monocyte recruitment. Because TRPC3 is the only TRPC member whose high constitutive, non-regulated activity has been shown to operate under either heterologous or native expression conditions (Trebak, Vazquez et al. 2003) those findings raised the question whether TRPC3 contributes to expression of VCAM-1 through regulated activity, constitutive activity, or both. In a follow up study using TNFα to induce VCAM-1 expression and a combination of real-time fluorescence and silencing RNA approaches, we conclusively showed that it is the constitutive function of TRPC3 which mediates most of the Ca\(^{2+}\) influx required for regulated expression of VCAM-1 in HCAECs (Smedlund and Vazquez 2008).

In most endothelial cells VCAM-1 expression is regulated, at the transcriptional level, by nuclear factor kappa B (NFκB)(Zhang 2008) and we have shown that this is also the case in HCAECs. NFκB activation involves its release from the inhibitory protein IkBα and then the subsequent translocation of NFκB to the nucleus where it modulates transcriptional activity of target genes. Release of NFκB from IkBα is preceded by phosphorylation of IkBα by IkBα kinases (IKKs) followed by its ubiquitination and proteasomal degradation (Tergaonkar 2006). Because in most cells types examined so far NFκB activation depends, directly or indirectly, on Ca\(^{2+}\) influx, and TNFα-induced VCAM-1 requires constitutive Ca\(^{2+}\) influx (Smedlund, Tano et al. 2010), we examined...
whether TRPC3, through its constitutive function, contributes to the mechanism by which NFkB modulates VCAM-1 expression in HCAECs. Interestingly, our studies showed that knockdown of TRPC3 in HCAECs drastically reduced the ability of TNFα to induce phosphorylation of IκBα and its upstream regulator IKKβ (Smedlund, Tano et al. 2010), and this correlated with an inhibition of IκBα degradation. These findings indicated for the first time that TRPC3 constitutive function is an obligatory component in the signaling driving TNFα-dependent activation of NFkB. In addition, we showed that TRPC3-mediated Ca²⁺ entry is fundamental to activate the calmodulin (CAM)/calmodulin kinase II (CAMKII) axis in a NADPH oxidase-dependent manner, and this signaling axis in turn activates NFkB (Smedlund, Tano et al. 2010). Importantly, our studies brought about a conceptually novel perspective on the role of TRPCs in cardiovascular disease, as they underscored for the first time, the potential pathological impact of upregulated expression of a TRPC channel endowed with high constitutive activity and how this may relate with pathological Ca²⁺-dependent signaling, independently of the canonical pathway driven by receptor stimulation. This is of particular interest to the field, as in those instances where TRPCs participate in mechanisms associated to inflammatory vascular disease (reviewed by us in (Tano, Smedlund et al. 2010)) their contribution relates to regulated, or receptor-dependent channel function, rather than constitutive activity. In vivo studies are underway using mouse models of atherosclerosis with genetically manipulated levels of endothelial TRPC3 in order to determine the potential impact of TRPC3 expression and constitutive function in the context of the molecular and cellular events that lead to atherosclerotic lesion development in the intact vessel.

3.2 TRPC3 and macrophage survival

As stated earlier (section 2.2) the balance between apoptotic macrophages and their clearance by resident phagocytes at the lesion site is determinant in the progression of the atherosclerotic lesion. Within this context, signaling events that modulate the survival rate of the macrophage have a tremendous impact on such balance, provided efferocytic properties of resident phagocytes remain unaffected. Recent studies in our laboratory have implicated non-regulated, constitutive Ca²⁺ influx in the signaling associated with macrophage survival. Two major pathways are essential for the survival of macrophages in the atherosclerotic lesions: the phosphatidylinositol-3-kinase (PI3K)/AKT axis and the NFkB route. In the PI3K/AKT pathway, macrophage survival signals (v.g., insulin-like growth factor, prostaglandin E2) acting through either receptor tyrosine kinases or G-protein coupled receptors induce activation of PI3K in the plasma membrane and the subsequent generation of 3’-phosphorylated phosphoinositides such as phosphatidylinositol 3, 4 bisphosphate/3,4,5 trisphosphate. These phosphoinositides allow for recruitment and activation of PDK-1 which then leads to full activation of AKT kinase. One of the major mechanisms of AKT-dependent survival takes place through AKT-mediated phosphorylation of the pro-apoptotic protein BAD, a member of the Bcl-2 family. Upon AKT-mediated phosphorylation, BAD releases the anti-apoptotic proteins Bcl-2 and Bcl-x, preventing mitochondrial release of cytochrome c and thus progression of the mitochondrial apoptotic pathway (Datta, Brunet et al. 1999). As for macrophage survival through the transcription factor NFkB, it is known that activation of NFkB exquisitely regulates the transcriptional status of several survival genes. Both of the survival pathways described
above are highly active in THP-1 derived macrophages (TDMs) upon exposure to the atherorelevant cytokine TNFα (Tano and Vazquez 2011). Notably, maneuvers that prevent constitutive Ca\(^{2+}\) entry through Ca\(^{2+}\) permeable channels drastically reduce the phosphorylation of IkBα, AKT and its downstream target BAD, with the subsequent increase in macrophage apoptotic rate (Tano and Vazquez 2011). In addition, when TDMs are pre-treated with pharmacological inhibitors of CAM and CAMKII, activation of survival signaling is prevented as efficiently as blockade of constitutive Ca\(^{2+}\) influx does. These findings indicated for the first time that activation of macrophage survival pathways depends, to a significant extent, on constitutive Ca\(^{2+}\) influx presumably through a mechanism involving the CAM/CAMKII axis (Tano and Vazquez 2011). A particularly interesting observation derived from those studies was that inhibition of PI3K function completely abrogated TNFα-dependent NFkB activation suggesting that the PI3K/AKT axis exerts a regulatory action on the NFkB pathway. Operation of such crosstalk has been demonstrated in cell types other than macrophages, where AKT-dependent transactivation of NFkB acts as an alternative AKT-dependent anti-apoptotic route independently of the AKT/BAD axis (Romashkova and Makarov 1999; Madrid, Wang et al. 2000; Bai, Ueno et al. 2009). In summary, our studies suggest that in human macrophages a CAM/CAMKII axis links constitutive Ca\(^{2+}\) influx to activation of AKT, which then serves as a signaling node to promote survival through NFkB and/or phosphorylation of BAD.

Macrophages from both human and mouse origin express TRPC3, and TRPC3 constitutive function has been shown to be operational in different cell types from these two mammalian species. These attributes make TRPC3 a great candidate to mediate the constitutive Ca\(^{2+}\) influx that supports the macrophage survival mechanisms described above. We indeed examined this possibility in recent studies that made use of bone-marrow derived macrophages obtained from mice globally deficient in TRPC3 (Tano, Smedlund et al. 2011). Macrophages derived from TRPC3 deficient bone marrow (TRPC3\(^{-/-}\)) exhibited a significant reduction in constitutive cation influx compared to TRPC3\(^{+/+}\) cells (Tano, Smedlund et al. 2011). Most importantly, the number of apoptotic macrophages in response to TNFα was significantly higher in TRPC3\(^{-/-}\) cultures than in those of TRPC3\(^{+/+}\) macrophages, indicating a diminished survival in macrophages lacking TRPC3. Importantly, these observations correlated very well with the activation status of survival signaling: the phosphorylation of IkBα, AKT and BAD was severely reduced in TRPC3\(^{-/-}\) macrophages (Tano, Smedlund et al. 2011). Altogether, these findings indicated that TRPC3 has an obligatory role in macrophage survival and that TRPC3 is likely to mediate the constitutive Ca\(^{2+}\) influx required for proper operation of survival signaling.

As described above, clearance of apoptotic macrophages by resident phagocytes at the lesion site is fundamental for appropriate inflammation resolution, and is a major factor in determining lesion cellularity. By means of an in vitro efferocytosis assay in which TRPC3\(^{+/+}\) and TRPC3\(^{-/-}\) were used as either phagocytes or apoptotic cells, we observed that the phagocytic function of TRPC3\(^{-/-}\) macrophages is drastically impaired when compared to that of TRPC3\(^{+/+}\) phagocytes; interestingly, apoptotic TRPC3\(^{-/-}\) cells seem to be poor substrates for phagocytosis regardless of the phagocyte’s TRPC3 expression status (Tano, Smedlund et al. 2011). Although additional studies are required to clarify TRPC3’s role in efferocytosis, these findings suggest a critical requirement for TRPC3 within the signaling associated to phagocytic activity and/or cell-cell recognition processes that underlie efferocytosis.
4. TRPC3 as a prospective target in atherosclerosis: Roadmap of an exciting TRiP

The rapid advance in elucidating signaling mechanisms associated to atherogenesis was enthusiastically perceived as an opportunity to develop anti-inflammatory strategies to manage the disease. However, the multifactorial nature of atherosclerosis makes such therapeutic strategies, often aimed at interfering on single targets, of limited efficacy and it is likely that multiple targeting is necessary to achieve clinically significant outcomes (Yonekawa and Harlan 2005; Preiss and Sattar 2007; Recio-Mayoral, Kaski et al. 2007). This is not surprising if we take in consideration the multifactorial nature of atherosclerosis and the diverse repertoire of signaling molecules and cell types that contribute to its pathogenesis. In that context, identifying new components of signaling events linked to monocyte recruitment and/or modulation of macrophage survival/apoptosis at the lesion site is of fundamental importance to move forward in the search for additional potential targets that could make those alternative therapeutic strategies a reality. Furthering our knowledge on the potential new roles of TRPC3, as well as other TRPC members, in atherogenesis can make a significant contribution to the search for new targets for the disease. Although ubiquitously expressed throughout tissues, TRPC3 on endothelial or macrophage surface could be used as a molecular target of relatively easy access for therapeutic and/or diagnostic purposes or may be exploited as a marker in non-invasive imaging, as it has been applied to other cell surface proteins (Kaufmann, Carr et al.; Saraste, Nekolla et al. 2009). The potential advantages of TRPC3 vs. traditional channel blockers as a prospective target for atherosclerosis has recently been discussed by us (Vazquez 2011). The exploration of TRPC3 as an atherorelevant signaling molecule is at its infancy, and several additional studies will be required to determine the impact of TRPC3 expression/function on atherorelevant events. The generation and characterization of new mouse models of atherosclerosis with genetically manipulated levels of TRPC3 in the atherorelevant cell or tissue of interest (i.e., conditional knockouts or transgenics) will be a unique contribution to that goal.

5. References


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