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Nitric Oxide-Releasing Aspirin in Atherothrombosis: A Remarkable Improvement of an Old Drug

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ABSTRACT

Anti-platelet treatment is now a first line therapeutic strategy in coronary atherosclerosis, and it is prescribed in almost all the high-risk patients. However, despite the wide use of aspirin against atherothrombosis, its side effects and especially those from the gastrointestinal tract do not allow its use in patients at high risk for gastrointestinal bleeding. Therefore, a newly developed drug, the nitric oxide (NO)-releasing aspirin, provides new hope for eliminating the side effects of the classic aspirin. NO releasing aspirin is consisted of an aspirin moiety and an NO-donating complex, leading to the release of NO preventing the local gastrointestinal bleedings. Further to its effects in the gastrointestinal tract, NO-releasing aspirin seems to be superior to classic aspirin, by providing both the antithrombotic effect of aspirin and the beneficial anti-atherogenic, anti-apoptotic and anti-thrombotic effects of NO at a vascular level. NO-releasing aspirin releases NO in specific cellular compartments, mimicking the endogenous NO synthesis. Although this new promising type of aspirin provides the hope for a global anti-atherothrombotic effect in all the high-risk patients, its clinical usefulness is still under evaluation. Despite the existing encouraging reports from basic and the first clinical trials, the drug is still at phase II, and it is still premature to state with confidence that it may replace the classic and well studied aspirin, in the fight against atherothrombosis.

Keywords: atherosclerosis, cardiovascular risk, NCX-4016, nitric oxide, thrombosis
Abbreviations: COX, cyclo-oxygenase; **CVD**, cardiovascular disease; **cGMP**, cyclic guanosine monophosphate; **FMD**, flow-mediated vasodilation; **GI**, gastrointestinal; **GTP**, guanosine triphosphate; **MI**, myocardial infarction; **NO**, nitric oxide; **NOS**, nitric oxide synthase; **PG**, prostaglandin; **TIA**, transient ischemic attacks; **TX**, thromboxane

CONTENTS

INTRODUCTION 38
MECHANISM OF ACTION OF ASPIRIN 38
SITE EFFECTS OF ASPIRIN 39
THE ROLE OF ASPIRIN IN CARDIOVASCULAR DISEASE (CVD) 40
Aspirin in primary prevention of CVD 40
Aspirin in secondary prevention of CVD 40
Aspirin in the acute phase 40
MECHANISM OF ACTION OF NITRIC OXIDE (NO)-RELEASING ASPIRIN 41
NO-RELEASING ASPIRIN AND GASTRIC BLEEDING 41
NO-RELEASING ASPIRIN AND ATHEROTHROMBOSIS 41
NO and cell apoptosis 41
NO and atherogenesis 41
The role of NO in platelets aggregation and thrombosis 42
DATA FROM CLINICAL AND BASIC STUDIES FOR NO-RELEASING ASPIRIN 42
CONCLUSION 43
REFERENCES 43

INTRODUCTION

For decades, salicylates from plant sources provided a folk remedy for pain and fever (Hayden 1975). In the mid 1800s, salicylic acid was synthesized in Europe, followed shortly thereafter by the synthesis of acetyl-salicylic acid (Vane *et al*. 1990), while many years later, acetyl-salicylic acid was developed as a commercial pharmaceutical product, which was called "Aspirin" (Vane *et al*. 1990). Despite its toxic effects on gastrointestinal tract (Derry *et al*. 2000) it became a first line agent against cardiovascular death, by preventing thrombotic coronary events (Patrono *et al*. 2001) and atherothrombosis in general (Patrono *et al.* 2005), further to its well-known anti-inflammatory properties (Vane *et al*. 1990). However, a newly developed drug, the nitric oxide (NO) releasing aspirin, provides new hope for eliminating the side effects of the classic aspirin. Although this new promising type of aspirin provides the hope for a global anti-atherothrombotic effect in all the high-risk patients, its clinical usefulness is still under evaluation.

MECHANISM OF ACTION OF ASPIRIN

The mechanism of action of aspirin and especially its effects on the prostaglandin (PG) synthesis have been studied extensively in the past. It is well known that the central en-

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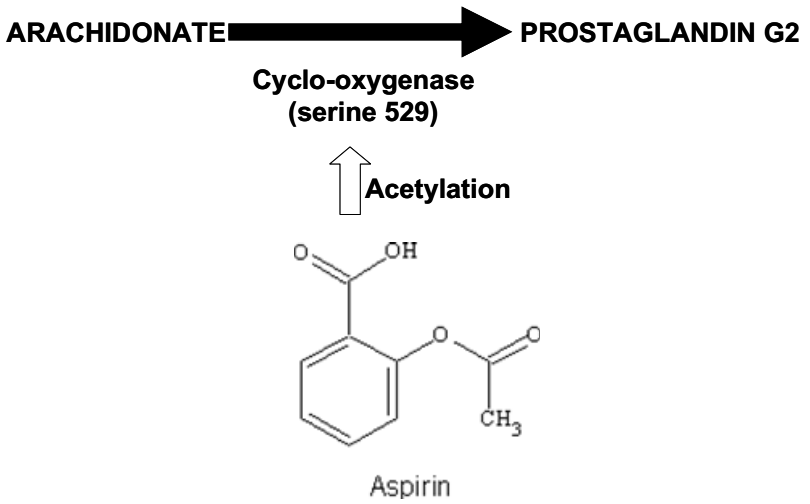

Fig. 1 Aspirin: Mechanism of action. Aspirin induces the acetylation of a single serine (residue 529) within the active site of cyclooxygenase, affecting the arachidonate/prostaglandin G2 equilibrium.

zyme of PG synthesis, PGH synthase, controls both cyclooxygenase (COX) (oxygenates/cyclizes arachidonate to PGG2) and peroxidase (reduces PGG2 to PGH2) activities (Miyamoto *et al*. 1976; Ohki *et al*. 1979; Roth *et al*. 1981). Aspirin affects PG synthesis in a specific way by blocking only the COX function of PGH synthase (Smith *et al*. 1971) while leaving other PG elements unaffected.

At a molecular level, aspirin blocks COX by acetylating the protein, and the reaction depends on both the intrinsic chemical properties of the drug and the affinity of the drug for the enzyme's active site (Roth *et al*. 1975a, 1975b, 1983). Aspirin "nonspecifically" acetylates a variety of proteins, lipids, and nucleic acids at millimolar concentration. In contrast, it acetylates COX in a highly "specific" fashion with the reaction going to completion within minutes at micromolar concentrations under mild conditions (Roth *et al*. 1975a, 1975b) The thousand-fold difference in aspirin concentrations needed for "nonspecific" versus "specific" acetylations reflects the affinity of the drug for a single serine (residue 529) (**Fig. 1**) within the active site of COX (Roth *et al*. 1983). Acetylation by aspirin of serine 529 within COX produces a covalent O-acetyl bond that resists hydrolysis under intracellular conditions. The "permanence" of acetylation results in the irreversible inactivation of platelet COX by aspirin (Roth *et al*. 1975a, 1975b). Thus, aspirin-modified platelets are affected for the rest of their circulating lifespans. Platelet and endothelial COX are equally sensitive to aspirin, as shown by direct experiments in intact cells (Jaffe *et al*. 1979). Although an other study comparing the enzyme from blood vessel microsomes with that of platelets suggested that the platelet enzyme was more sensitive to aspirin (Burch *et al*. 1978) most researchers consider the endothelial and platelet enzymes to be equivalent in their response to aspirin particularly because they appear to be identical proteins that are encoded by the same gene (Funk *et al*. 1991).

Aspirin is very popular for its antithrombotic, analgesic, antipyretic and antirheumatic actions. However effective analgesic, antipyretic and antirheumatic doses of aspirin are much higher than those needed to inhibit platelets (Patrignani *et al*. 1982). One small dose of oral aspirin "permanently" impairs the function of all available platelets, with the effect lasting several days (Burch *et al*. 1978; Patrignani *et al*. 1982; FitzGerald *et al*. 1983).

The gradual recovery of platelet function after a dose of aspirin is the converse of aspirin's initial "cumulative" effect and reflects the appearance in the circulation of new, unaffected platelets that were formed in the marrow after the ingestion of the drug. The "anucleate" nature of platelets and the covalent nature of aspirin dependent acetylation combine to produce the "permanence" of the aspirin effect on platelet PG synthesis (Burch *et al*. 1978; FitzGerald *et al*. 1983; Reilly *et al*. 1987). Selective elimination of the thromboxane (TX)-A2 pathway by aspirin causes a clear-cut but modest decrease in platelet function and provides a limited but definite antithrombotic effect. In contrast, nucleated cells such as endothelium can replenish their supply of COX after aspirin treatment by synthesizing new enzyme, and this ability to recover from aspirin treatment contributes to the reduced aspirin sensitivity of these cells as compared with platelets (Patrignani *et al*. 1982; FitzGerald *et al*. 1983).

SITE EFFECTS OF ASPIRIN

Unfortunately, long-term administration of aspirin is accompanied by an increased risk of site effects such as gastric ulceration and decreased renal and hemostatic function (Wolfe *et al*. 1999). Other unusual affects of aspirin are urticaria and related idiosyncratic reactions (Samter *et al*. 1968). Aspirin induces gastrointestinal ulceration and bleeding by a mechanism that may involve both inhibition of PG synthesis and direct damage to gastric and intestinal mucosa through contact with ingested drug tablet (Graham *et al*. 1990; Gabriel *et al*. 1991). This unfavourable effect of aspirin is revealed from the inhibition of COX, which mediates the decreased synthesis of proinflammatory mediators (such as PG and TX) induced by aspirin. Inhibition of COX derived cytoprotective PG at gastric mucosa explains this side effect of aspirin. Clinical and endoscopic data show the dose-dependency of aspirin's GI (gastrointestinal) toxicity (Prichard *et al*. 1989) and antithrombotic trials with aspirin corroborate this fact. However, the gastric mucosa appears to adapt to aspirin and various preparations of aspirin (enteric coating, buffered preparations) and two therapeutic modalities (misoprostol, ranitidine) may moderate the GI toxicity of the drug (Graham *et al*. 1990). Furthermore suppression of renal PG synthesis with attendant vasostimulation is unlikely to occur with low doses of aspirin, reiterating the argument to use lower doses of the drug in thrombosis. The bleeding disorder induced by aspirin is usually small and goes unrecognized in hemostatically normal individuals as a mild increase in mucosal bleeding (Weiss *et al*. 1968; Mustard *et al*. 1970). The antihemostatic and antithrombotic effects of aspirin are inseparable because both result from platelet inhibition. Any aspirin dose that exceeds a low "threshold" level will block platelet PG synthesis and produce both bleeding risks and potential antithrombotic benefits. The potential clinical importance of the hemostatic defect resulting from aspirin was suggested by the primary prevention trials in which an increase in life-threatening intracerebral hemorrhage was observed in men taking aspirin (Peto *et al*. 1988). The same toxicity has been observed in some primary prevention trials (ETDRS Investigators 1992) but not in others (Manson *et al*. 1991) and it is not seen in secondary trials in which an increase in intracerebral bleeding may be obscured by a concomitant decrease in thrombotic, occlusive strokes (Hirsh *et al*. 1992; Sherman *et al*. 1992). The incidence of

bleeding caused by the dose independent, antihemostatic effect of aspirin can not easily be measured. However the potential threat of increased intracerebral bleeding should be a strong restriction to the unlimited and uncritical use of aspirin for the primary prevention of thrombosis.

THE ROLE OF ASPIRIN IN CARDIOVASCULAR DISEASE (CVD)

Aspirin in primary prevention of CVD

To date, 6 major primary prevention trials of aspirin have been conducted involving 47,293 subjects on aspirin and 45,580 not on aspirin or placebo: British Doctors' Trial (BDT), Physicians' Health Study (PHS) (Steering Committee of the Physicians' Health Study Research Group 1989), Thrombosis Prevention Trial (TPT) (The Medical Research Council's General Practice Research Framework 1998), Hypertension Optimal Treatment (HOT) study (Hansson *et al*. 1998), Primary Prevention Project (PPP), (Collaborative Group of the Primary Prevention Project 2001) and the Women's Health Study (WHS) (Ridker *et al*. 2005), In all these trials patients were randomized to aspirin and had follow-up durations ranging from 4 to 10 years. The PHS was the first study to demonstrate that aspirin reduced the risk of a first MI (myocardial infarction) among apparently healthy men (Hennekens *et al*. 1988; Steering Committee of the Physicians' Health Study Research Group 1989). This trial was terminated early based on the unanimous recommendations of the Data and Safety Monitoring Board primarily because of the extreme, statistically significant reduction in risk of first MI (Hennekens *et al*. 1988; Steering Committee of the Physicians' Health Study Research Group 1989). The smaller BDT used an open design and its results showed no significant cardioprotective benefits of aspirin. Because of its small sample size, this trial could not have detected even a 44% reduction in risk for first MI, as was shown in the PHS. The use of aspirin and/or warfarin in the primary prevention of ischemic heart disease (IHD) was examined in the TPT (The Medical Research Council's General Practice Research Framework 1998). Aspirin use reduced all IHD by 20%, predominantly because of a 32% reduction in nonfatal events. However, aspirin had no significant effect on fatal events and little or no benefit on stroke. The combination of warfarin and aspirin led to a 34% reduction of all IHD but increased hemorrhagic and fatal strokes. The randomized HOT trial examined the role of low-dose aspirin therapy in the prevention of CVD in patients with hypertension (Hansson *et al*. 1998). The use of aspirin significantly reduced the incidence of major cardiovascular events. Aspirin conferred the greatest cardioprotective effect against fatal and nonfatal MI (32%) but had no significant effect on the incidence of stroke (Hansson *et al*. 1998). The PPP trial assessed aspirin and vitamin E therapy in primary prevention of cardiovascular events in people with 1 or more major cardiovascular risk factors (Collaborative Group of the Primary Prevention Project 2001). The trial was terminated early based on the evidence of aspirin's benefits documented in earlier trials (Hebert *et al*. 2000). In the most recent study (WHS) (Ridker *et al*. 2005), 39,876 initially healthy women were randomly assigned to receive 100 mg of aspirin on alternate days or placebo and they were then monitored for 10 years for a first major cardiovascular event. In this large, primary-prevention trial among women, aspirin lowered the risk of stroke without affecting the risk of MI or death from cardiovascular causes.

In summary, the primary prevention trials indicate that aspirin therapy conclusively reduces the risk of first MI, but the results are less conclusive with regard to stroke and vascular death (Eidelman *et al*. 2003).

Aspirin in secondary prevention of CVD

In 1988, the Antiplatelet Trialists' Collaboration (ATC) published their first meta-analysis of 25 randomized trials of about 25,000 survivors of MI, stroke, or transient ischemic attacks (TIA) involving prolonged antiplatelet therapy in the reduction of important vascular events (nonfatal MI, nonfatal stroke, and vascular death) (Antiplatelet Trialists' Collaboration 1988). By 1994, the second ATC analyzed 145 randomized trials involving approximately 70,000 high-risk and 30,000 low-risk patients, as well as 29 trials comparing different antiplatelet regimens involving another 10,000 high-risk patients (Antiplatelet Trialists' Collaboration 1994). The third ATC included 287 trials: 197 involving 135 000 patients randomized to antiplatelet therapy or control and 90 trials that compared different antiplatelet regimens among 77,000 patients (Antithrombotic Trialists' Collaboration 2002). The vast majority of these trials tested aspirin as the antiplatelet regimen. Antiplatelet therapy, primarily with aspirin, clearly and consistently afforded significant protection against CVD in these trials in all high-risk groups (Antithrombotic Trialists' Collaboration 2002). Patients given aspirin had a 25% reduction in serious vascular events, as a result of 34% reduction in nonfatal MI, 25% reduction in nonfatal stroke, and 17% reduction in vascular death (Antithrombotic Trialists' Collaboration 2002). In addition, there was no increased risk of nonvascular death. Aspirin use results in a significant, 15% decrease in CVD mortality among patients who have survived a wide range of prior occlusive events, and a significant, 25% reduction in important vascular events (Antithrombotic Trialists' Collaboration 2002). In the 1980s, the US Food and Drug Administration (FDA) approved aspirin for the treatment of patients with prior MI and unstable angina, as well as men with prior TIAs (Hennekens *et al*. 1994; Eidelman *et al*. 2003). In 1998, the FDA expanded the indications for aspirin to include women with prior TIAs, patients with prior occlusive stroke or chronic stable angina, and those who have undergone revascularization procedures (Eidelman *et al*. 2003).

Aspirin in the acute phase

The Second International Study of Infarct Survival (ISIS-2) examined the effects of aspirin administered during acute MI. At 35 days, patients randomized to aspirin had significant reductions in vascular mortality. Furthermore patients receiving aspirin had significant reductions in nonfatal reinfarction and nonfatal stroke, with no increased incidence of hemorrhagic stroke or GI bleeding. During acute MI, uncoated aspirin is preferable. Patients using enteric-coated aspirin are instructed to crush or chew the tablets to achieve a rapid clinical antithrombotic effect. In 1997, the FDA approved the use of aspirin, in doses ranging from 160 to 325 mg/day, for the treatment of acute MI. A benefit-to-risk analysis suggests that for every 1000 patients who have an acute MI, aspirin initiated within 24 hours of onset of symptoms would prevent 23 premature deaths, with no increase in cerebral hemorrhage (Hennekens *et al*. 1994). It is currently estimated that wide use of aspirin in patients having an acute MI would prevent 5000 to 10,000 premature deaths annually in the United States (Hennekens *et al*. 1997). The Chinese Acute Stroke Trial (CAST) examined the effects of aspirin in 21 106 patients with suspected acute ischemic stroke (CAST 1997). The results of the trial demonstrated a significant reduction in mortality during the treatment period and a 12% reduction in risk of death or nonfatal stroke at 4 weeks, compared with placebo. In addition, patients in the aspirin group had significantly fewer recurrent ischemic strokes than did those in the placebo group. Aspirin was also associated with 2:1000 rate of hemorrhagic strokes in patients with suspected ischemic stroke (CAST 1997). CAST investigators estimated that 10,000 premature deaths and new-onset nonfatal stroke or MI could be prevented annually through the early adminis-

tration of aspirin to 1 million patients with ischemic stroke, and continued therapy after hospital discharge would further reduce morbidity and mortality (CAST 1997).

MECHANISM OF ACTION OF NITRIC OXIDE (NO)-RELEASING ASPIRIN

NO-releasing aspirins (such as NCX-4016) are designed based on a simple but brilliant idea: to combine in a single molecule, the most widely used agent in CVD, aspirin, and a donor of probably the most important signaling molecule in human vessels, NO. NCX 4016 is a hybrid molecule consisted of the ester group between the carboxylic function of acetylsalicylic acid, the hydroxylic function of the NO-donating moiety, and the nitric ester group that is responsible for the NO donation. The first ester group can be easily hydrolyzed by esterases, but this step is not followed by immediate release of NO. In fact, after oral administration of NCX 4016, the unchanged NO-donating moiety has been detected in a circulating metabolite of NCX 4016 produced by esterases. NO is released from this metabolite via slowly formed bioactive intermediates such as *S*-nitrosothiols (Carini *et al*. 2004). However the underlying mechanisms in the formation of *S*-nitrosothiols from NCX 4016 have not yet clearly elucidated.

The release of NO from NCX-4016 *in vivo* seems to be time- and concentration- dependent, since it possibly shares a common pathway with glycerol trinitrate, as implied by the presence of bi-directional cross-tolerance at the level of cGMP stimulation between these two molecules (Grosser and Schröder 2000). It is likely that this pathway depends on the cytochrome P450 system (Schröder *et al*. 1992). A recent *in vitro* study on different NO-donating aspirin isomers also suggested the possible involvement of the cytosolic glutathione *S*-transferase in NO release (Gao *et al*. 2005). An interesting observation is that salicylate and NO-derived species have a similar formation pattern, suggesting that both acetylosalicylic acid and NO moieties are simultaneously released and exert their beneficial effects at the same time (Carini *et al*. 2001; Bolla *et al*. 2006). It has also been demonstrated that endothelial cells internalize NCX 4016 and that the release of NO from NCX 4016 occurs in the same cell compartments as the endogenous production of NO from L-arginine (Fiorucci *et al*. 2002).

NO-RELEASING ASPIRIN AND GASTRIC BLEEDING

As noted above long-term administration of aspirin is accompanied by an increased risk of site effects such as gastric ulceration and bleeding. This is the result of both the inhibition of PG synthesis and the direct damage to gastric and intestinal mucosa through contact with ingested drug tablet (Graham *et al*. 1990; Gabriel *et al*. 1991). Although NCX 4016 inhibits the synthesis of gastric PG E2 it did not show any gastrointestinal complications when administered at high doses in rats (Fiorucci *et al*. 1999). In the same study it was shown that NO-aspirin spares the gastric mucosa and inhibits caspase activity through cGMP-dependent and -independent pathways.

NO-release from NCX 4016 provides one reasonable explanation for the absence of gastric damage. The role of NO in gastric safety has been extensively studied in the past (Brown *et al*. 1992; Whittle *et al*. 1993). NO regulates the mucosal blood flow (Whittle *et al*. 1993) and stimulates mucus secretion (Brown *et al*. 1992). This explains the cytoprotective effect of NO on the mucosal cells. Other studies, on NO vascular activities have also shown that NCX 4016 induces an increase in mucosal blood flow, thereby accounting for local/systemic gastric protection (Takeuchi *et al*. 1998), and reduces leukocyte adherence to postcapillary mesenteric venule vessel walls (Wallace *et al*. 1997).

On the other hand NCX 4016 has antiapoptotic effects on gastric mucosal cells and this could be through the modulation of caspase activity (Fiorucci *et al*. 1999). This is probably the second mechanism by which gastric mucosa is protected. It has also been shown in animal models that the pathological state does not affect the beneficial effects of NCX 4016 on the gastric mucosa (Tashima *et al*. 2000; Kato *et al*. 2001; Napoli *et al*. 2002). Furthermore, while aspirin increases the mucosal ulcerogenic response and impairs the healing response of gastric ulcers, NCX 4016 was found not to impair the healing response (Takeuchi *et al*. 1998; Ukawa *et al*. 1998). In addition, the combination of NCX 4016 with a selective COX-2 inhibitor did not increase the risk of gastric damage in either animal (Wallace *et al*. 2004) or human gastric mucosa (Fiorucci *et al*. 2003). In conclusion we could say that *in vitro* and *in vivo* studies have clearly shown that NCX 4016 has a protective role on mucosal cells and eventually its use does not provoke any complication from the gastrointestinal system such as bleeding or ulceration.

NO-RELEASING ASPIRIN AND ATHEROTHROMBOSIS

NO is the signalling molecule responsible for several physiological and pathophysiological processes. It is synthesised from L-arginine by three isoforms of the enzyme nitric oxide synthase (NOS). It has been demonstrated that NO controls vascular smooth muscle tone, inhibits platelet and inflammatory cell adhesion and activation, and is a transmitter at non-adrenergic non-cholinergic synapses (Moncada *et al*. 1991; Quinn *et al*. 1995). Furthermore, NO can also modulate apoptosis, or programmed cell death, in a variety of cell types, including human inflammatory cells (Taylor *et al*. 2003). The pathways by which NO exerts many of its actions is via activation of the enzyme soluble guanylate cyclase (Moncada *et al*. 1991) and resultant conversion of guanosine 5′-triphosphate (GTP) to the second messenger 3′, 5′-cyclic guanosine monophosphate (cGMP) (Ignarro *et al*. 1999). In addition NO can act via cGMP-independent pathways in various systems, particularly during the inhibition of platelet aggregation and regulation of inflammatory cell apoptosis (Gordge *et al*. 1998; Sogo *et al*. 2000; Ward *et al*. 2000; Crane *et al*. 2002).

NO and cell apoptosis

NO can be both pro- and anti-apoptotic, depending on local concentrations and the specific cell type (Quinn *et al*. 1995; Kim *et al*. 1999; Taylor *et al*. 2003). The pro- and anti-apoptotic actions of NO have been well documented in many cell systems. For example, high concentrations of either exogenous or endogenous iNOS-derived NO have been shown to induce apoptosis in murine macrophage cell lines (Albina *et al*. 1993; Sarih *et al*. 1993). On the other hand, low concentrations of NO generated from the spontaneous NO donors, SPER/NO and DEA/NO, reduced the rate of neutrophil apoptosis (Taylor *et al*. 2001).

Consequently, we can say that lower concentrations of NO produced by the constitutive endothelial and neuronal isoforms of NOS (eNOS and nNOS) are cytoprotective, whilst supraphysiological concentrations produced by the inducible NOS isoform (iNOS) trigger cell death (Nicotera *et al*. 1997). This can be explained, by the free radical nature of NO and hence the ease with which it will react with other radicals, particularly reactive oxygen species, present in the milieu to form various NO-related species *in vivo*.

NO and atherogenesis

Atherogenesis is a multi-factorial condition with a complicated aetiology. The underlying causes of atherogenesis remain largely unknown, although a critical early stage is thought to be an insult to the endothelium, either physical or through oxidative stress (Ross 1999a, 1999b). Initially, the injured endothelium becomes dysfunctional and production of NO by eNOS decreases, promoting vasocons-

triction and platelet and inflammatory cell adhesion. Secondly, a protective inflammatory response is triggered. However, depending on the nature and duration of the insult, this protective response becomes excessive and over a period of years, comes to constitute the disease process itself (Ross 1999a, 1999b). Thus, accumulation of inflamematory cells, (monocytes and macrophages) leads to further plaque growth. However, the plaque is dynamic and inflammatory cells are constantly turning over within the core. It is well established that apoptotic cells, particularly macrophages, are present in atherosclerotic plaques in both human and animal models of the disease (Bjorkerud and Bjorkerud 1996; Haunstetter and Izumo 1998). Because apoptotic cells are ingested by phagocytes without initiating any further proinflammatory response, it has been suggested that apoptosis may represent a mechanism to regress the plaque. NO is a particularly promising candidate for this strategy because, as well as to it's pro-apoptotic actions, it has several other powerful anti-atherogenic characteristics including a powerful inhibitory effect on platelet and inflammatory cell activation (Moncada *et al*. 1991; Armstrong 2001). For example, administration of L-arginine (the substrate for NOS) to hypercholesterolemic rabbits increases the number of apoptotic macrophages in intimal lesions by three fold. This increase in apoptosis was associated with a regression of the plaque, suggesting that manipulation of the NOS pathway may well represent a therapeutic approach to resolving the inflammatory response in the vessel wall (Wang *et al*. 1999). However, care must by exercised when considering this approach because NO is also known to induce apoptosis in smooth muscle cells (Labelle *et al*. 2004). Loss of cells from the fibrous cap during the latter stages of atherosclerosis may destabilize the plaque and promote rupture (Kockx and Knaapen 2000).

The role of NO in platelets aggregation and thrombosis

Endothelial NO has been shown to have important antiplatelet actions (Azuma *et al*. 1986; Radomski *et al*. 1987). By activating guanylyl cyclase, inhibiting phosphoinositide 3-kinase, impairing capacitative calcium influx, and inhibiting COX-1, endothelial NO limits platelet activation, adhesion, and aggregation. Platelets are also an important source of NO, and this platelet-derived NO pool limits recruitment of platelets to the platelet-rich thrombus.

It has been demonstrated that in addition to the undergoing oxidation to nitrite and nitrate, reacting with superoxide anion to form peroxynitrite, and reacting with heme iron to form the charge-transfer complex required to activate guanylyl cyclase, NO and oxygen or peroxynitrite can react with thiols to form *S*-nitrosothiols (Stamler *et al*. 1992; Kelm *et al*. 1999). These latter compounds serve as stable reservoirs of NO, which can be transferred to and from protein-bound pools18 by *trans*-*S*-nitrosation reactions (Scharfstein *et al*. 1994; Liu *et al*. 1998). Other studies also suggest that *S*-nitrosothiols can be stored by platelets and released during heterotypic cellular interactions (Hirayama *et al*. 1999).

N-Acetyl-L-cysteine potentiates the antiplatelet effect of endothelial NO, (Stamler *et al*. 1989) and this action can be mimicked by the *S*-nitroso-*N*-acetyl-L-cysteine (Mendelsohn *et al*. 1990) which inhibits both thrombin-induced and U-46619 (a stable TX-A2 analogue)-induced expression of platelet surface P selectin (a granule protein), CD63 (a lysosomal protein), and the calcium-dependent active conformation of the heterodimeric fibrinogen-binding integrin glycoprotein IIb/IIIa (Michelson *et al*. 1996). This is associated with suppression of intracellular calcium flux and demonstrable reduction in both the affinity (2.7-fold increase in *K*d) and number (50% decrease) of fibrinogen-binding sites on the platelet surface (Mendelsohn *et al*. 1990). Inhibition of cytosolic calcium flux with exposure to strong platelet agonists like thrombin or U46619 seems to be a consequence of inhibition of capacitative calcium influx resulting from enhanced sarcoplasmic reticulum/endoplasmic reticulum calcium-ATPase–dependent refilling of calcium stores (Trepakova *et al*. 1999). *S*-Nitroso-*N*-acetyl-L-cysteine–dependent reduction in fibrinogen binding is dose-dependent and correlates strongly with NO-dependent activation of platelet guanylyl cyclase and cGMP accumulation (Mendelsohn *et al*. 1990).

Activation of platelets is associated with activation of another important signaling pathway, the phosphoinositide 3-kinase (PI3-kinase) pathway. It has been shown that nitrovasodilators can induce platelet disaggregation (Stamler *et al*. 1989) and platelet PI3-kinase renders platelet aggregation irreversible. Furthermore the effect of the *S*-nitrosothiol *S*-nitroso-glutathione on platelet PI3-kinase were also studied and it was shown that the NO donor inhibits the thrombin receptor–activating peptide stimulation of PI3-kinase activity associated with tyrosine-phosphorylated proteins in immunoprecipitates and of p85/PI3-kinase associated with the src family kinase member lyn (Pigazzi *et al*. 1999). The activation of PI3-kinase complexed with lyn requires the activation of lyn itself and other tyrosine kinases, and inhibition of this process by the NO donor is cGMP-dependent and likely involves inhibition of the dephosphorylation of lyn required for its activation.

DATA FROM CLINICAL AND BASIC STUDIES FOR NO-RELEASING ASPIRIN

Evidence suggests that NCX-4016 may have most of the beneficial effects of NO in human vasculature, in addition to the beneficial aspirin-mediated effects (Antoniades *et al*. 2007). Experimental evidence (Yu *et al*. 2002) suggests that NCX-4016 can reduce vascular inflammation and prevent apoptosis during vascular remodeling associated with neointimal thickening, as a result of its NO-releasing capacity. In another animal model (Emanueli *et al*. 2004), pretreatment with NO-releasing aspirin derivative stimulated reparative angiogenesis and prevented apoptosis and oxidative stress. In animal models, (Gresele and Momi 2006) NCX 4016 protected from platelet thromboembolism, prevented restenosis in atherosclerosis-prone animals, protected the heart from ischemia/reperfusion injury, and induced neoangiogenesis in critically ischemic limbs. Moreover, it displayed little or no gastric toxicity and appeared to protect stomach from noxious stimuli, including aspirin.

Clinical trials, suggest that NCX-4016 may be beneficial by preventing restenosis after percutaneous intervention (Napoli *et al*. 2002), having also significant antihypertensive effects (Muscara *et al*. 2001). There is also evidence that further to the expected inhibition of platelet TX synthesis and aggregation, it also down-regulates tissue factor and inhibits interleukin-6 and monocyte chemoattractant protein-1 expression after chronic treatment, having in this way additional antiatherogenic properties in humans (Fiorucci *et al*. 2004). In addition, a most recent study showed that two novel furoxan–aspirin hybrids drugs effectively inhibit collagen-induced platelet aggregation and the relative contribution of NO to the inhibitory effect was dependent on the characteristics of the specific furoxan involved (Turnbull *et al*. 2006).

Furthermore, in a gastroscopic study in healthy volunteers (Fiorucci *et al*. 2003) in which a 7-day treatment with NCX 4016 (400 or 800 mg b.i.d.) was compared with a 7-day treatment with aspirin (200 or 400 mg b.i.d.), platelet aggregation induced by a low arachidonic acid concentration as well as arachidonic acid -induced platelet TX production were inhibited by both drugs to the same extent at the highest dose. Serum TX-B2 levels, as an expression of the production capacity of blood platelets, were also largely and significantly inhibited by NCX 4016 at both doses, although not to the same extent as equimolar aspirin. In addition, salicylate plasma levels were increased in both aspirin- and NCX 4016-treated volunteers, while a significant increase in the NO metabolites nitrite and nitrate in plasma,

which confirmed NO-delivery *in vivo*, was evident only for the NCX 4016-treated group (Fiorucci *et al*. 2003).

Another interesting issue is the effect of NO-releasing aspirin on venous by-pass grafts vasomotion. Although a beneficial effect of the drug on by-pass grafts was demonstrated (Lorusso *et al*. 2007), there is a lot more to be done before NCX-4016 is accepted to be beneficial in CABG patients. It remains to be proven that its vasodilatory/antithrombotic effect is translated to a decreased postoperative vein graft failure. Despite the inconsistent results from randomized clinical trials and the lack of complete angiographic follow-up, it seems that aspirin itself may be beneficial in reducing graft occlusion at 12 months after CABG (Okrainec *et al*. 2005). On the other hand, there is no data examining whether nitrates may have any beneficial effect on grafts patency (Okrainec *et al*. 2005).

In another trial using a human clinical model of endotoxemia following LPS infusion, pre-treatment with NCX 4016 prevented a significant rise in soluble P-selectin, while pre-treatment with aspirin did not (Marsik *et al*. 2002). In addition, the pre-treatment with NCX 4016 markedly reduced plasma levels of some of the cytokines, such as IL-6, IL-8, interferon-γ and monocyte chemotactic protein-1 all of which play an important role in the inflammatory component of atherosclerosis (Fiorucci *et al*. 2002).

Additionally a recent trial (Gresele *et al*. 2004), has been carried out in patients with intermittent claudication in order to assess comparatively the effects of a 1-month treatment with NCX 4016 and a conventional antithrombotic dose of aspirin on effort-induced endothelial dysfunction. Endothelial function was assessed by flow-mediated vasodilation (FMD) of the brachial artery before and immediately following exercise on a treadmill. After 28 days of treatment, FMD preceding the exercise routine was not significantly different from baseline; however, following the exercise routine, the impairment of FMD was abolished in the NCX 4016 group, but not in the aspirin group. The same investigator (Gresele and Momi 2006) has also shown that NCX 4016 inhibits platelet activation *in vitro* more effectively than aspirin, inhibits smooth muscle cell proliferation, exerts an endothelial cell protective activity and suppresses the function of several inflammatory cells potentially involved in atherothrombosis.

Very recent data also suggest that NCX 4016 may be superior that classic aspirin, in preventing the acute endothelial dysfunction induced by exercise in patients with intermittent claudication (Gresele *et al*. 2007), providing one of the first reports supporting the superiority of this new compound against aspirin.

Other ongoing phase II studies assess the ability of NCX 4016 to reduce proteinuria in diabetic patients and the early marker of the atherosclerotic involvement of the renal vascular bed, and/or the acute platelet and inflammatory changes induced by short-term hyperglycemia in type II diabetes (Gresele *et al*. 2003).

CONCLUSION

The beneficial role of aspirin in cardiovascular disease is now widely accepted. NO-releasing aspirin has a unique pharmacological profile obtained through its COX inhibitory and NO-donating properties. This activity profile of NO-releasing aspirin is encouraging and suggests that the drug can be used for treating clinical conditions where inflammatory mediators are pivotal factors in the disease progression, such as in the atherosclerotic state, acute coronary syndromes, restenosis after angioplasty and peripheral vascular disorders. This new form of aspirin, has the advantage of causing less gastrointestinal side effects, since the release of NO in the gastric mucosa has a protective effect against the aspirin-induced gastric injury. Therefore, it could be administered with success to those patients requiring aspirin intake but not being treated with this drug due to contra-indications associated with gastrointestinal ulceration/bleedings.

Although NO-releasing aspirin seems to be a promising therapeutic strategy against atherothrombosis, the drug is still in phase II clinical trials, and several questions about its safety and its optimum dosage need to be answered before it becomes a tool in the hands of clinical practitioner. This drug needs to be tested for its safety and its efficacy, and its effectiveness has to be proven in a clinical setting, by large-scale clinical trials. Thus, it is still premature to state with confidence that it may replace the classic and well studied aspirin, in the fight against atherothrombosis.

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