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Angiogenesis and Inflammation Signaling Are Targets of Beer Polyphenols on Vascular Cells

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ABSTRACT

Emerging evidence indicates that chronic inflammation and oxidative stress cluster together with angiogenic imbalance in a wide range of pathologies. In general, natural polyphenols present health-protective properties, which are likely attributed to their effect on oxidative stress and inflammation. Hops used in beer production are a source of polyphenols such as xanthohumol (XN), and its metabolites isoxanthohumol (IXN) and phytoestrogen 8-prenylnaringenin (8PN). Our study aimed to evaluate XN, IXN, and 8PN effects on angiogenesis and inflammation processes. Opposite in vitro effects were observed between 8PN, stimulating endothelial and smooth muscle cell (SMC) growth, motility, invasion and capillary-like structures formation, and XN and IXN, which inhibited them. Mouse matrigel plug and rat skin wound-healing assays confirmed that XN and IXN treatments reduced vessel number as well as serum macrophage enzymatic activity, whereas 8PN increased blood vessels formation in both assays and enzyme activity in the wound-healing assay. A similar profile was found for serum inflammatory interleukin-1β quantification, in the wound-healing assay. Our data indicate that whereas 8PN stimulates angiogenesis, XN and IXN manifested anti-angiogenic and anti-inflammatory effects in identical conditions. These findings suggest that the effects observed for individual compounds on vascular wall cells must be carefully taken into account, as these polyphenols are metabolized after in vivo administration. The modulation of SMC proliferation and migration is also of special relevance, given the role of these cells in many pathological conditions. Furthermore, these results may provide clues for developing useful therapeutic agents against inflammation- and angiogenesis-associated pathologies. J. Cell. Biochem. 111: 1270–1279, 2010.

KEY WORDS: ANGIOGENESIS; INFLAMMATION; ENDOTHELIAL CELLS; SMOOTH MUSCLE CELLS; BLOOD VESSELS; POLYPHENOLS; XANTHOHUMOL; ISOXANTHOHUMOL; 8-PRENYLNARINGENIN; BEER

In adulthood, the angiogenic process is usually localized, as in wound repair and tissue regeneration, and is self-limited in time as happens in ovulation (days), wound healing (weeks), and placentation (months). Pathological angiogenesis, inversely, may develop during years.

The growing life span in western countries has promoted an increase in the proportion of elderly people in the growing population, with consequent increased needs for health care. Cardiovascular diseases (CVD) together with cancer comprise the vast majority of deaths among the elderly people [Hotamisligil, 2006]. Obesity has also emerged as a major public health problem due to its high prevalence and association with increased risk for development of type 2 diabetes mellitus and CVD [Hotamisligil, 2006; Monteiro, 2009; Soares, 2009]. Despite their distinct etiopathogenesis, CVD, cancer, diabetes, and obesity are now being considered angiogenesis-dependent diseases, associated with a chronic inflammation state and oxidative stress that together with hypoxia are the main stimuli of the angiogenic process originating unstable and leaky vessels [Costa et al., 2007; Folkman, 2007]. Although inflammation is an essential process responsible for defending the organism against pathogens, it may also have adverse effects on surrounding tissues, especially when it persists over time [Imhof and Aurrand-Lions, 2006]. Inflammatory cells secrete cytokines and growth factors, which promote angiogenesis, and a strong association between factors involved in inflammation and angiogenesis, playing a role in endothelial cells (EC) biology has been described [Imhof and Aurrand-Lions, 2006; Costa et al., 2007]. Conversely, angiogenesis sustains inflammation by providing oxygen and nutrients for the cell metabolic requirements at inflammatory sites [Costa et al., 2007]. Gaining insights into

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angiogenesis and the role of angiogenic factors in several unrelated diseases will enable a better understanding of the potential of angiogenic modulators, as well as the need for preventive and therapeutic strategies both inhibiting and promoting angiogenesis [Folkman, 2007].

Epidemiological and experimental evidence indicates that diets rich in plant-derived foods and several beverages like red-wine, tea, and beer offer a protective effect against several pathologies interfering both in inflammatory and angiogenic processes [Gerhauser, 2005a; Oak et al., 2005; Soares and Azevedo, 2007]. These properties have been mainly attributed to the anti-oxidant and anti-inflammatory effects of polyphenolic compounds [Biesalski, 2007; Stevenson and Hurst, 2007].

Beer is a highly consumed beverage around the world. Hop-derived supplements and beer contain several polyphenols. Xanthohumol (XN), which has received most attention in recent years can be converted to isoxanthohumol (IXN), and to the potent phytoestrogen 8-prenylnaringenin (8PN) during beer production and in vivo metabolism [Nikolic et al., 2005, 2006; Bolca et al., 2007]. Beer polyphenols have generally been described as potent anti-oxidative, anti-inflammatory, and anti-carcinogenic molecules, being, thus, possible contributors to the prevention of pathologies with high incidence and mortality rates in the western world [Stevens and Page, 2004; Gerhauser, 2005a,b; Cho et al., 2008; Magalhaes et al., 2009]. Conversely, the phytoestrogenic properties described for 8PN, namely as an estrogen receptor agonist, enables the putative use of this molecule in pathological conditions such as osteoporosis or menopause-associated complications [Milligan et al., 2002].

Despite the increasing number of studies regarding beer polyphenols, the actions of these compounds in EC and specially vascular SMC are not elucidated. Herein, we investigated the effects of XN, IXN, and 8PN in angiogenesis and inflammation, focusing our attention in both the two types of vascular wall cells—EC and SMC. The possibility of the metabolization of XN into IXN and IXN into 8PN, increases the interest in studying the effects of these compounds using the same experimental conditions. In vivo models able to confirm the angiogenic and inflammatory modulation were also used for the three polyphenols.

MATERIALS AND METHODS

CELL CULTURES

Human umbilical vein endothelial cells (HUVEC) and human aortic smooth muscle cells (HASMC) were purchased in ScienceCell Research Labs (San Diego). HUVECs were cultured in M199 medium (Sigma-Aldrich, Portugal) supplemented with 20% fetal bovine serum (FBS) (Invitrogen Life Technologies, Scotland, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies), 0.01% heparin (Sigma-Aldrich), and 30 μg/ml endothelial cell growth supplement (Sigma-Aldrich), and maintained at 37°C in a humidified 5% carbon dioxide atmosphere. Cells were seeded on plates coated with 0.2% gelatin (Sigma-Aldrich) and allowed to grow. HASMC were cultured in Dulbeco's modified Eagle's medium. HASMC cultures were supplemented with 10% FBS and 1% penicillin/streptomycin and cultured at 37°C in a humidified 5% carbon dioxide atmosphere. Cells were kept between passages 2 and 8 for every experiment. XN, 8PN (Sigma-Aldrich), and IXN (Alexis, Switzerland) were dissolved in ethanol and then added to cell culture medium at a concentration of 0.1–10 μM, established according to the viability assays performed. Polyphenols and vehicle (ethanol) were added to cell cultures in medium supplemented with 2% FBS and 1% penicillin/streptomycin. Control cells were incubated with vehicle (ethanol). Ethanol concentrations were kept below 0.1% in every culture.

CELL VIABILITY

HUVEC and HASMC were allowed to grow until 70–80% confluence and then incubated with 0.01–20 μM XN, IXN, 8PN or ethanol for 24 h. After the incubation period, cells were washed twice with phosphate-buffered saline solution and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [Guerreiro et al., 2007]. Briefly, cells were incubated with MTT solution at a final concentration of 0.5 mg/ml for 3 h and then lysed in dimethylsulfoxide. Absorbance was measured at 540 nm, and the background absorbance, measured at 660 nm, was subtracted. All samples were assayed in duplicate and at least in three independent experiments, and the mean value for each experiment was calculated. The results are given as mean (±SEM) and are expressed as percentage of control, which was considered to be 100%.

CELL APOPTOSIS

HUVEC and HASMC (1×10^4 cells/ml) were grown on glass coverslips and incubated with different concentrations (0.1–10 μM) of tested compounds (XN, IXN, and 8PN) for 24 h. TUNEL assay was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Switzerland), as reported before [Soares et al., 2004; Rocha et al., 2007]. The percentage of stained cells was evaluated by counting the cells stained with TUNEL (apoptotic cells) divided by the total number of nuclei stained with DAPI (Roche Diagnostics) at a 200× magnification field.

CELL PROLIFERATION

HUVEC and HASMC cultures (1×10^4 cells/ml) were established on glass coverslips following treatment procedures with 0.1–10 μM polyphenols (XN, IXN, and 8PN) for 24 h. Cell proliferation analyses were carried out using cellular incorporation of 5′-bromodeoxyuridine (BrdU), a thymidine analogue. After incubation with BrdU solution at a final concentration of 0.01 mM for 24 h, the number of proliferating cells (positive for BrdU), after immunohistochemistry methods using anti-BrdU-specific antibodies (BrdU In-Situ Detection Kit, BD Biosciences Pharmingen), were evaluated at the microscope, according to the manufacturer's instructions and as previously described [Rocha et al., 2007].

INVASION CAPACITY

The invasive cell behavior in the presence of 0.1–10 μM XN, IXN, and 8PN was quantified in vitro using a double-chamber assay by counting the number of cells that invaded a Transwell BD-Matrigel® basement membrane matrix inserts (BD Biosciences, Belgium),

according to manufacturer's instructions. FBS was used as a chemoattractant. Results represent the ratio between invading cells in polyphenol-treated cultures compared to invasion in control cultures for the same initial amount of cultured cells.

CAPILLARY-LIKE STRUCTURES FORMATION

Cells were cultured on growth factor reduced-Matrigel® (GFR-Matrigel®) (BD Biosciences, Belgium)-coated plates for 24 h as previously described [Soares et al., 2004]. Briefly, HUVEC were cultured on GFR-Matrigel®-coated plates for 24 h, in medium containing 1–10 μM polyphenols or vehicle. When cultured on Matrigel®, EC assemble into capillary-like structures. The number of cord-like structures was then counted at an inverted microscope. Each cord portion between the ramifications was considered one cord unit. Mean values were obtained by evaluating the whole cultures of each well under the same treatment. Treatments were performed as described above. A semi-quantitative measurement of cord formation in GFR-Matrigel® cultured HUVEC was developed as previously described [Soares et al., 2004].

IN VIVO STUDIES

Animal experiments were conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals).

MATRIGEL PLUG ASSAY

A mixture of Matrigel® and heparin without (negative control, C−) or with recombinant VEGF (positive control, C+) and 10 μM polyphenols (XN, IXN, and 8PN), was subcutaneously inoculated into C57BL/6 mice (purchased at Charles River, Wilmington, MA). The animals were euthanized after 7 days, the matrigel plug was removed, weighed, photographed and the amount of hemoglobin (Hb) in the homogenized plug was measured, as described below. Mice blood was also collected for evaluation of inflammatory factors.

SKIN WOUND-HEALING ASSAY

Wistar rats (Charles River), 8- to 12-week-old were used and kept individually in their cages during the study. After general anesthesia, the rat dorsal skin was shaved and full skin-thickness longitudinal incisions (1.5 cm) were created and the wound edges surgically sutured at 0.5 cm intervals. Polyphenols (XN, IXN, and 8PN) or vehicle (ethanol + water, 1 + 5; C) were administered topically (50μL of a 50μM solution), daily as observed in the literature [Malinda et al., 1998; Koczulla et al., 2003; Monteiro et al., 2008]. Rats were examined daily for wound-healing progression. After 7 days wound tissue was collected for histological studies and blood used for inflammatory factors evaluation. Skin wound tissue specimens were fixed in 10% neutral-buffered formalin and paraffin-embedded. Histological and immunohistochemistry analyses were performed in 5-μm tissue sections.

HEMOGLOBIN DETERMINATION

The Hb content of the plug was evaluated after homogenization of the plug in a water–heparin solution, which was then centrifuged at 1,500*g* for 15 min at 20°C. The supernatant (100 μl) was used to measure the Hb content according to the Drabkin's method (Sigma-Aldrich) at 540 nm.

SERUM ANALYSES

IL1β Measurement. Interleukin (IL) 1β was quantified in mice and rats serum by ELISA (IL-1β-EASIA kit, BioSource, Nivelles, Belgium) according to the manufacturer's instructions.

Determination of *N*-Acetylglucosaminidase Activity. The *N*-acetylglucosaminidase (NAG) enzyme is present at high levels in activated macrophages. Inflammation can be evaluated by measuring the levels of the lysosomal NAG enzyme in the serum. Serum was incubated for 10 min at 37°C with 100 μl of p-nitrophenyl-*N*-acetyl-β-D-glucosaminide solution in a 96-well plate. The reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.6) and the substrate hydrolysis was measured at 405 nm.

IMMUNOHISTOCHEMISTRY ANALYSIS

Microvessel density (MVD) was evaluated in each formalin-fixed paraffin-embedded wounded tissue section by immunohistochemistry. Tissue slides were incubated with an anti-von-Willebrand Factor (vWF) antibody (Santa Cruz Biotechnologies, CA). Capillaries were then counted in the three tissue sections, for each animal, and normalized to the total area of the tissue section. Negative controls were carried out by omission of the primary antibody in tissue sections expressing the marker.

STATISTICAL ANALYSES

Every cell experiment was performed at least in three independent experiments. Quantifications are expressed as mean (SEM) and as percentage of control, which was considered to be 100%. Statistical significance of difference between various groups was evaluated by analysis of variance (ANOVA) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $P < 0.05$.

RESULTS

MICROMOLAR CONCENTRATIONS OF POLYPHENOLS AFFECT HUVEC AND HASMC VIABILITY

Cell cytotoxicity was first analyzed by MTT assay in the two cell cultures upon treatment with 0.01–20 μM of XN, IXN, or 8PN (Fig. 1). Viability of HUVEC was only decreased by the higher concentrations of XN (39.40 ± 3.86% decrease, for 20 μM) and IXN (19.38 ± 5.38% decrease, for 10 μM and 33.59 ± 7.05% decrease, at 20 μM). Conversely, an increase in the number of viable cells was found after incubation with 8PN, in a dose-dependent manner, reaching statistical significance at 20 μM (40.75 ± 9.82% increase). HASMC viability was significantly reduced by XN at higher concentrations as well (39.91 ± 15.82% decrease, for 10 μM and 93.91 ± 0.81% decrease, at 20 μM). No statistical differences were observed when these cells were treated with IXN or 8PN at any concentration.

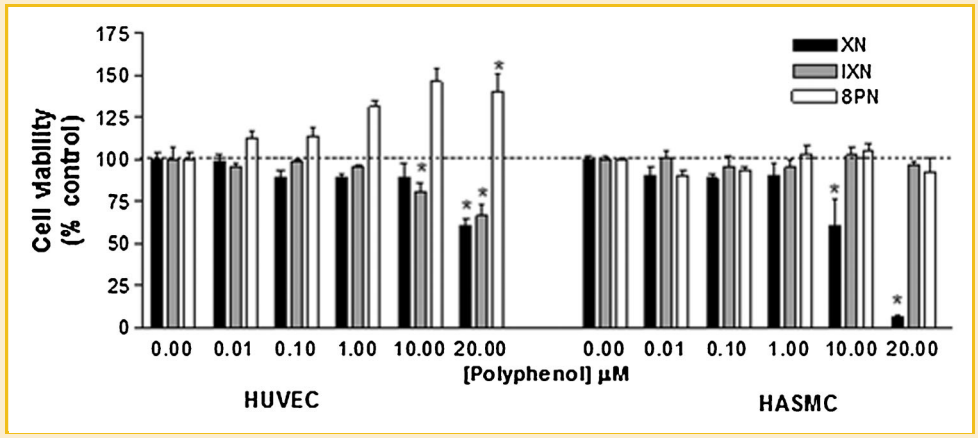

Fig. 1. Effect of polyphenols on HUVEC and HASMC viability. Cell viability after incubation for 24 h with 0.01–20 μM xanthohumol (XN), isoxanthohumol (IXN), or 8-prenylnaringenin (8PN) or vehicle (0.00) was evaluated by MTT assay. Results are means ± SEM of independent experiments ($4 \leq n \leq 7$) and are expressed as percentage of control. $^{*}P < 0.05$ versus control.

As the decrease in cell viability at 20 μM was too high for some of the tested compounds, we proceeded the study using 10 μM as the higher concentration tested.

XN, IXN, AND 8PN DISTINCTLY AFFECT HUVEC AND HASMC APOPTOSIS, PROLIFERATION, AND INVASION

In order to understand whether the observed cell viability alterations upon polyphenol treatments were due to their actions on apoptosis or cell growth, we next investigated the potential apoptotic and proliferative activity of the three compounds at 0.1, 1, or 10 μM concentration. Incubation of both cell cultures with XN and IXN for 24 h resulted in a significant increase in apoptosis, reaching statistical significance for 10 μM concentration of both compounds in HUVEC cultures (69.88± 21. % increase for XN and 104.51 ± 17.02% increase for IXN) and in HASMC cells (100.82 ± 46.60% increase for XN and 53.55 ± 22.72% increase for IXN) (Fig. 2A). Inversely, treatment with 8PN reduced the percentage of apoptotic cells in both cell types (70.15 ± 9.09% decrease in HUVEC and 50.69 ± 6.69% decrease in HASMC), presenting a greater effect on HUVEC (Fig. 2A).

In contrast, incubation with 0.1–10.0 μM XN or IXN for 24 h significantly decreased cell growth in HUVEC (69.20 ± 5.89% decrease for XN and 65.71 ± 10.79% decrease for IXN treatments, at 10 μM) and in HASMC (82.14 ± 4.67% decrease for XN and 37.37 ± 4.80% decrease for IXN, at 10 μM) as illustrated in Figure 2B. Interestingly, 8PN resulted in a significant alteration of HUVEC proliferation (69.72 ± 18.24% increase at 10 μM), whereas no significant difference was seen in HASMC when compared to vehicle treated cells (Fig. 2B).

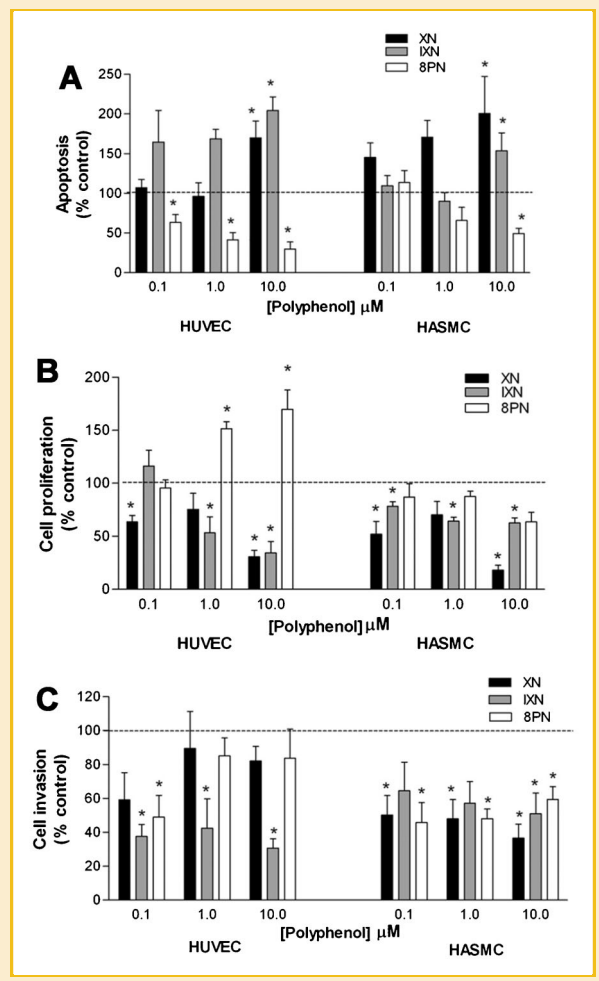

Fig. 2. Effect of polyphenols in HUVEC and HASMC apoptosis, proliferation and invasion. Cells were incubated for 24 h with 0.1–10 μM XN, IXN, or 8PN or vehicle. A: Apoptosis was evaluated by TUNEL assay. Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. B: The percentage of proliferative cells was examined by the ratio between BrdU-stained cells and hematoxylin-stained nuclei in every culture. C: The percentage of invading cells relative to the initial amount of cells cultured using a double-chamber assay. Results are means ± SEM of independent experiments ($4 \leq n \leq 8$) and are expressed as percentage of control. $^{*}P < 0.05$ versus control.

Cell motility and extracellular matrix invasion are fundamental steps within the angiogenic process. So, we next examined the effects of the three polyphenols on invasion capacity using a double chamber assay. As illustrated in Figure 2C, both XN (63.47 ± 8.30% reduction at 10 μM) and 8PN (40.55 ± 7.48% reduction at 10 μM) significantly reduced HASMC invasive capacity at the three concentrations analyzed. Although IXN showed a tendency towards decreased cell invasion, an effective decrease was only observed after incubation with the highest concentration of IXN (10 μM) (48.96 ± 12.15% reduction at 10 μM). IXN treatments of HUVEC resulted in a significant decrease in the ability to invade (69.72 ± 5.37% reduction for 10 μM) (Fig. 2C).

CAPILLARY-LIKE STRUCTURES FORMATION

To form a new blood vessel, EC must differentiate and reorganize, assembling into vascular capillary structures. HUVEC are able to assemble into highly branched capillary-like structures when cultured on GFR-Matrigel®. Therefore, we next examined whether XN, IXN, or 8PN were able to affect de novo the formation of in vitro capillary-like structures. Incubation of HUVEC cultures on GFR-Matrigel®-coated plates with XN or IXN at the concentration of 10 μM resulted in unconnected structures exhibiting loose edges, with many undifferentiated cells (Fig. 3A). Interestingly, organized and interconnected tubes were obtained after incubation with 10 μM 8PN (Fig. 3A). Quantitative analysis revealed a decrease in the number of capillary-like structures when HUVEC were treated with XN (26.64 ± 4.31% decrease) or IXN (31.33 ± 5.36% decrease), reaching statistical significance for 10μM concentration (Fig. 3B). Remarkably, 8PN-treated cultures exhibited a significant enhancement in the number of capillary-like structures (58.90 ± 4.34% increase) compared to control (Fig. 3B).

Altogether, these findings indicate that XN and IXN exhibit anti-angiogenic effects, whereas 8PN seem to trigger an opposite behavior in both cell cultures.

XN AND IXN DIMINISHED ANGIOGENESIS AND INFLAMMATION, WHEREAS 8PN INCREASED ANGIOGENIC PROCESS IN BOTH MOUSE MATRIGEL PLUG AND RAT WOUND-HEALING ASSAYS

To further examine the effects of the three compounds in angiogenesis and inflammation in a more accurate way, we assessed mouse matrigel plug neovascularization assay. As illustrated in Figure 4A,B, VEGF-containing matrigel (positive controls) presented extensive neovascularization. When matrigel implants in the presence of recombinant VEGF were mixed with either XN or IXN before inoculation, vascular development was strongly inhibited (Fig. 4A,B), with a higher inhibitory response for IXN (92.3 ± 2.32% decrease), identical to the negative controls, in which no VEGF was added. On the other hand, the plugs implanted with 8PN showed a robust angiogenic response identical to positive controls (79.98 ± 15.19%), as highlighted by the red color distributed in the whole plug (Fig. 4B).

Interestingly, analysis of the serum inflammatory enzyme NAG activity in these mice revealed that systemic inflammation decreased in the presence of XN or IXN implanted matrigel (67.40 ± 6.08% decrease for XN and 78.38 ± 2.30% decrease for IXN) (Fig. 4C). Surprisingly, implanted matrigel containing 8PN, which led to angiogenic stimulation similar to positive control, showed a reduction in systemic macrophage activity as revealed by NAG activity assay (69.28 ± 2.25% decrease).

Skin wound healing is a process involving the formation of new extracellular matrix, cell infiltration, and tissue remodeling. Two fundamental physiological conditions are deeply implicated in this process: inflammation and angiogenesis. After a preliminary experiment in the same conditions as described above, with daily monitoring of the wounds and histological evaluation of the inflammatory cytokines and vessel formation (data not shown), we decided to investigate the effect of the studied polyphenols in neovascularization and serum inflammatory markers in rat skin wounds as previously reported [Malinda et al., 1998; Koczulla et al., 2003]. Higher concentrations of polyphenols (50 μM) were used in

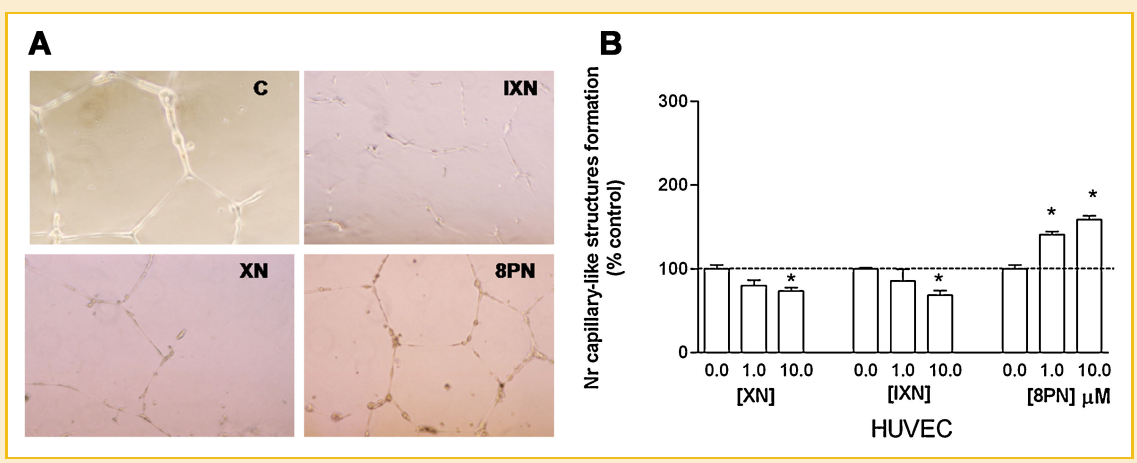

Fig. 3. XN and its metabolites, IXN and 8PN, differently affect the assembly of capillary-like structures. HUVEC were grown on GFR-Matrigel® and incubated with 1 or 10 μM XN, IXN, or 8PN or vehicle (C or 0.0) for 24 h. A: Capillary-like structures formation, after treatment with 10 μM polyphenols, visualized under a phase-contrast microscope. Figures are representative of the whole cultures. Magnification: 200×. B: Semi-quantification of capillary-like structures assembly. Results are means ± SEM of independent experiments ($3 \leq n \leq 5$) and are expressed as percentage of control. *$P < 0.05$ versus control. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

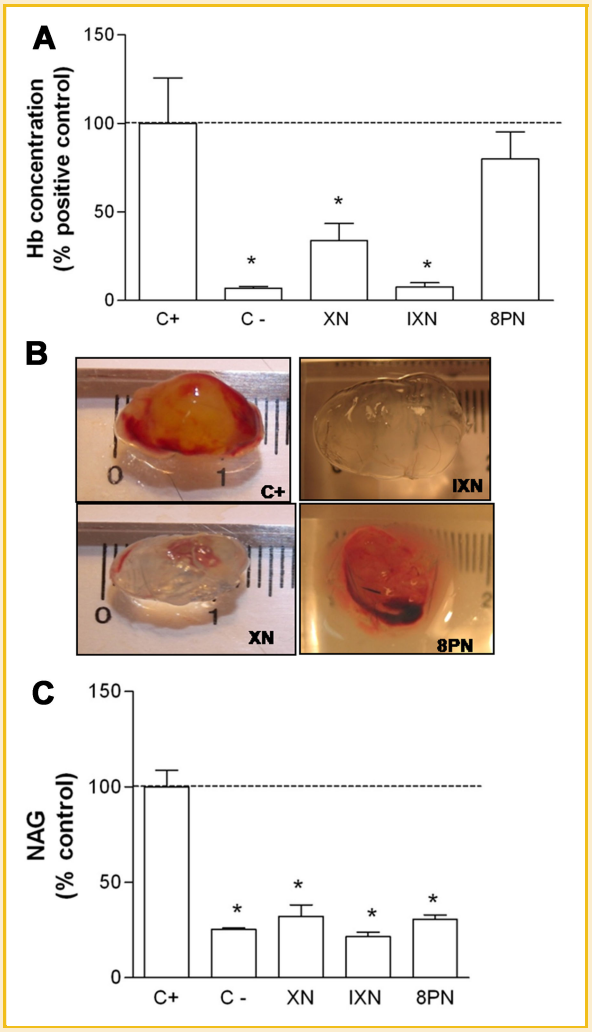

Fig. 4. In vivo evaluation of angiogenesis and inflammation using a mouse matrigel plug assay. A mixture of Matrigel® and heparin without vascular endothelial growth factor (VEGF) (negative control, C−); or of Matrigel®, heparin and VEGF (positive control, C+) and Matrigel®, heparin, VEGF and 10 μM XN, IXN, or 8PN was injected subcutaneously into C57BL/6 mice. A: Quantification of the hemoglobin (Hb) amount in the homogenized plugs by Drabkin's method. B: Representative images of macroscopic visualization of Matrigel plugs. XN and IXN diminished vessel formation in the plug, while 8PN increased it. C: Determination of *N*-acetylglucosaminidase (NAG) activity in mice serum 7 days after plugs implantation. Results are means ± SEM of independent experiments ($5 \leq n \leq 10$) and are expressed as percentage of control. $^{*}P < 0.05$ versus control. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

this in vivo assay because the compounds were used topically. Externally, healing process seemed completed on day 7 post-injury, with no differences observed in the wound area after a 7-day treatment with XN, IXN, or 8PN relative to vehicle. However, the corresponding histological sections used for immunohistochemical MVD quantification in the remodeled tissue revealed that upon treatments with XN and IXN the width of the granulation tissue formed at the incision site was thinner when compared to the corresponding area in controls (Fig. 5A). In contrast, 8PN treatment led to increased thickness of granulation tissue in certain areas (Fig. 5A). This apparent increase in granulation tissue observed upon 8PN treatment was accompanied by an increasing number of blood vessels in the area, contrary to what was observed after XN and IXN treatments, as accomplished by immunohistochemistry analyses (Fig. 5B). XN and IXN topic administration resulted in a decrease in MVD in the vicinity of the incision area (42.46 ± 9.71% decrease for XN and 50.46 ± 6.95% decrease for IXN) (Fig. 5C). Inversely, treatment with 8PN substantially increased the number of vessels assembled when compared to control (114.36 ± 2.42% increase). In addition, the activity of NAG enzyme in these rats serum was higher than in controls, revealing an increased systemic inflammatory status in rats treated with 8PN (28.67 ± 4.66% increase), whereas a significant lower inflammation for treatments with XN and IXN were observed (44.25 ± 6.20% decrease for XN and 22.72 ± 2.04% decrease for IXN) (Fig. 6A). These latter findings were further corroborated by an identical profile obtained for IL1β determination in rat sera (Fig. 6B). Higher concentration of tested polyphenols (50 μM) in this in vivo assay was used, when comparing polyphenol concentrations used in in vitro assays for EC and SMC treatments (10 μM). As this is an in vivo assay bioavailability and metabolization of these compounds must be taken into account, which justifies the application of higher doses in the skin treatments. Other authors observed no toxic effects with 100 μM ingestion of XN [Monteiro et al., 2008].

DISCUSSION

The present study investigated the effect of three beer polyphenolic compounds in angiogenesis and inflammation. We were able to demonstrate that overall, XN and IXN exerted anti-angiogenic effects both in cell cultures as well as in the in vivo angiogenic established models. In contrast, 8PN had the opposite effect, enhancing angiogenesis by acting on several angiogenic steps. These findings were in general accompanied by systemic inflammatory actions in mouse matrigel plug and rat wound-healing assays.

Our in vitro findings enabled us to identify which steps of the angiogenic process were targeted by each polyphenol. XN and IXN presented broad anti-angiogenic actions. Cell growth was compromised by XN and IXN in both cell cultures. Other authors have described similar growth effects in tumor cell lines [Colgate et al., 2007; Dell'Eva et al., 2007] and in EC [Bertl et al., 2004; Albini et al., 2006]. In addition, both compounds activated programmed cell death mechanisms in both vascular wall cells studied (HUVEC and HASMC) as well. XN and IXN also affected HUVEC and HASMC invasion ability and prevented the formation of capillary-like structures, implying their already established action on EC differentiation into cord structures [Bertl et al., 2004; Albini et al., 2006; Negrão et al., 2007]. Furthermore, the fact that these polyphenols also target SMC proliferation and migration, as revealed in this study, provides new evidence for the use of this agent in pathological situations exhibiting SMC hyperplasia, such as atherosclerosis or restenosis.

Inversely, 8PN exhibited anti-apoptotic effects in both cell cultures, but strikingly it stimulated HUVEC while it did not change HASMC proliferation. These results are not in accordance with the

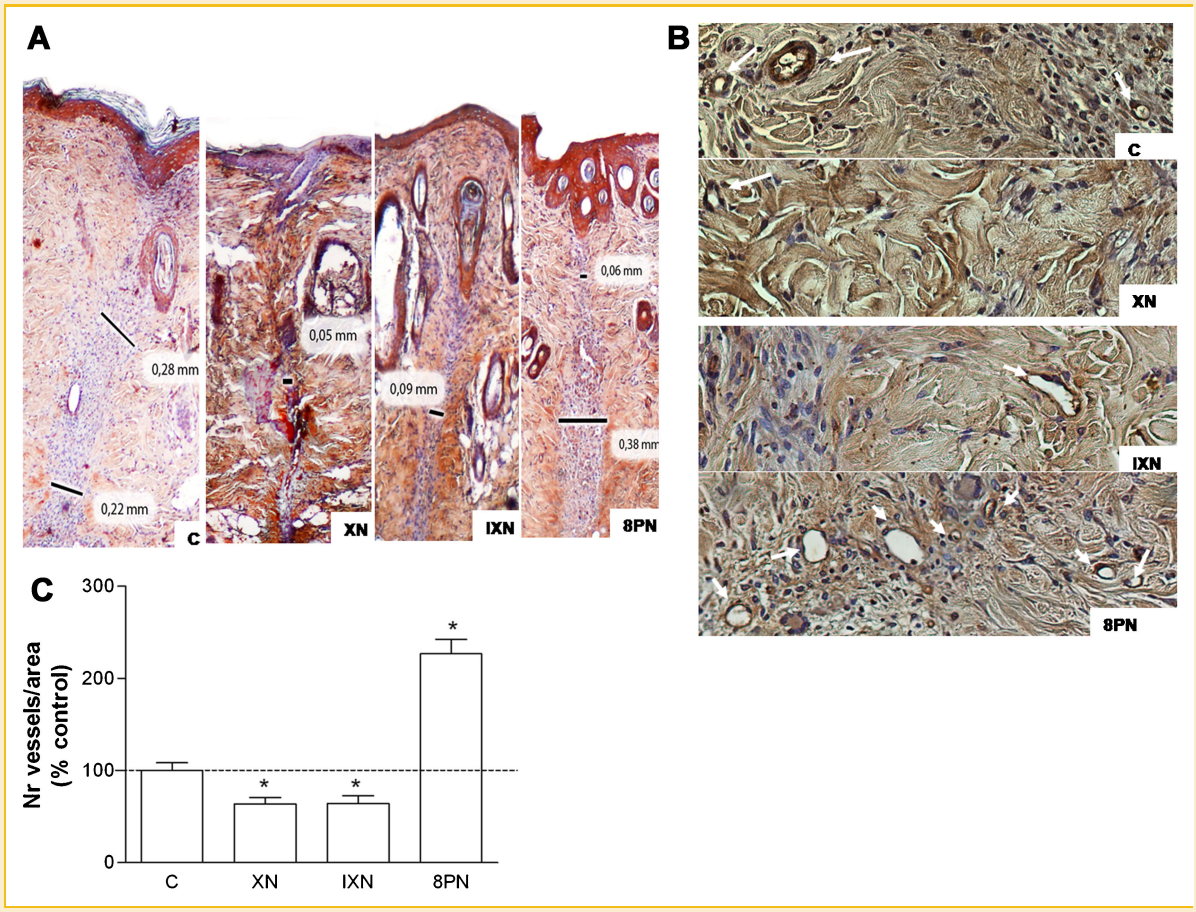

Fig. 5. In vivo skin wound-healing assay. Longitudinal incisions were created on the dorsal surface of the rats and 50 μM XN, IXN, 8PN or vehicle (C) were administered topically, daily. After 7 days, wounded tissue was collected for angiogenesis evaluation. A: Hematoxylin-stained micrographs of wound tissue sections, highlighting different thickness (black bar) of granulation tissue fulfilling the incision with different treatments (magnification: 40×). B: Wound tissue section micrographs from controls or rats treated with polyphenols, using von-Willebrand Factor (vWF) for evaluation of blood vessels (immunostaining, magnification: 200×). Arrows indicate blood vessels. C: Quantification of blood vessels present in three tissue sections, for each animal, and normalized to the total area of the tissue section. Results are means ± SEM of independent experiments ($4 \leq n \leq 7$) and are expressed as percentage of control. $^{*}P < 0.05$ versus control. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

previous reports in the literature [Pepper et al., 2004], attributing in vitro anti-angiogenic effects for 8PN in EC. Brunelli et al. [2007] suggested a biphasic action on cell growth for 8PN, showing estrogenic properties and increasing proliferation in hormone responsive cells at concentrations below 10 μM, while inhibiting proliferation at higher concentrations. Estrogens are established angiogenic promoters [Soares et al., 2003, 2004], rendering this molecule a putative pro-angiogenic agent. Furthermore, despite invasiveness capacity was not highlighted by the double chamber assay used, the number of in vitro cord structures increased significantly when HUVEC were treated with 8PN, supporting its pro-angiogenic effects (Fig. 3). Nevertheless, the mechanisms by which 8PN acts remain unknown.

Interestingly enough, XN and IXN significantly prevented VEGF-induced formation of de novo microvessels on the matrigel plug assay (Fig. 4), confirming that these two agents may inactivate VEGF signaling pathways. Although some studies have already highlighted the anti-angiogenic effect of XN, to our knowledge, this is the first report on the anti-angiogenic effects of IXN in vivo.

Chronic inflammation and angiogenesis are two joint partners. Inflammatory mediators produced by immune cells target EC to produce angiogenic factors, but those cells can directly release high amounts of distinct angiogenic factors. On the other hand, angiogenesis sustains inflammation, by providing oxygen and nutrients for the metabolic needs of the cells present at the inflammatory site as well as by enabling extravasation of immune cells [Costa et al., 2007]. Transgenic mice overexpressing VEGF show enhanced angiogenesis and immune responses [Xia et al., 2003], highlighting the existence of a complex coordination between blood vessels and immune cells [Costa et al., 2007]. Accordingly, mice implanted with XN and IXN-containing matrigel plugs presented decreased NAG macrophage enzyme activity in serum. These findings imply that the polyphenols retained inside the matrigel plug may exert systemic anti-inflammatory effects.

Unexpectedly, serum macrophage activity was also reduced by 8PN (Fig. 4C). In fact, matrigel plug assay is a well-established model to evaluate angiogenesis within the matrigel, but it is also a relatively artificial one. 8PN may directly or indirectly interfere with

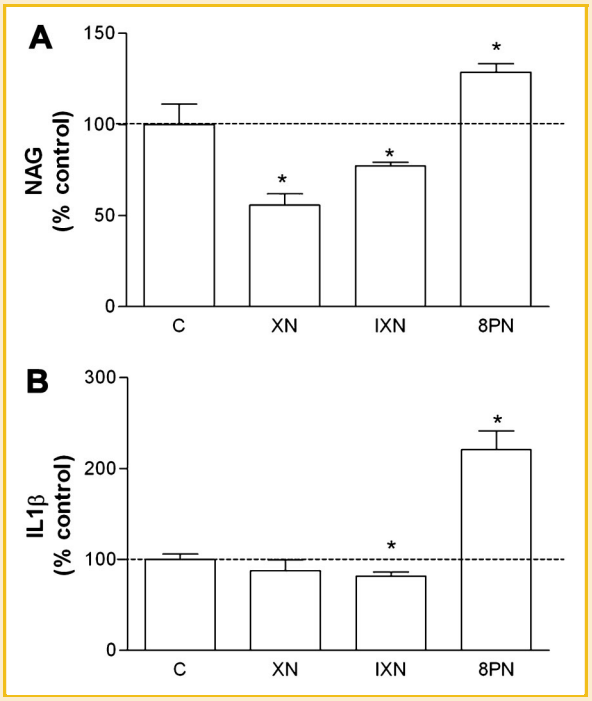

Fig. 6. In vivo rat skin wound-healing assay. Longitudinal incisions were created on the dorsal surface of the rats and 50 μM XN, IXN, 8PN or vehicle (C) were administered topically, daily. After 7 days, blood was collected for inflammation evaluation. A: Serum *N*-acetylglucosaminidase (NAG) activity. B: IL1β levels in rats serum. Results are means ± SEM of independent experiments ($4 \leq n \leq 7$) and are expressed as percentage of control. $^{*}P < 0.05$ versus control.

the release of specific stromal cell or matrix-derived factors which prevent inflammation.

A more physiological and accurate model for angiogenesis and inflammation evaluation is the skin wound-healing assay. No visible differences between the treatments in the time or external aspect of the healing process were found. But the histology of the injured area was effectively different. While XN and IXN efficiently diminished the development of the inflammatory and angiogenic processes in the skin incision, 8PN strongly stimulated both processes. The anti-inflammatory properties of XN are already extensively described [Cho et al., 2008; Monteiro et al., 2008; Magalhaes et al., 2009]. NFκB, a central regulator of the inflammatory process, is one of the known repressed pathways by this compound as well as IL1β production [Monteiro et al., 2008]. Again, we did not find any previous reports regarding the IXN effects in inflammation. Interestingly, we observed reduced thickness in the granulation tissue within the incision area upon topic treatment with XN and IXN. This is primarily attributed to their local effects in modulating angiogenesis and inflammation. A very recent study described the existence of a H_2O_2 gradient, just preceding the movement of the neutrophils towards the wound [Niethammer et al., 2009]. Initial H_2O_2 gradient expansion may be prevented by local application of these polyphenols. 8PN, on the other hand, enhanced vessel formation and increased inflammatory systemic markers (NAG activity and IL1β expression) in an apparent contradiction to previously described in vivo inhibition of angiogenesis by 8PN, in the chorioallantoic membrane model, where 8PN seemed to affect both vessel length and caliber [Pepper et al., 2004]. Yet, small capillaries were still present and less organized in the same study, consistently with our results that point towards a pro-angiogenic profile of 8PN.

Particular attention must be given to these three polyphenols, as they displayed opposite effects on vascular wall cells. Concerning their bioavailability, in hop cones, XN is the most abundant prenylated chalcone but in hop-derived products, generally IXN predominates over XN and 8PN [Gerhauser, 2005a]. Although polyphenols intestinal absorption may be rather limited, recent studies indicate that XN can be metabolized to IXN in the stomach [Nikolic et al., 2005]. Further, intestinal IXN may be converted in 8PN by intestinal bacteria [Possemiers et al., 2008] and by human liver microsomes [Nikolic et al., 2005]. Therefore, upon oral consumption of beer, or even XN, a mixture of the three compounds will be present in the human organism. The final outcome is likely dependent on the tissue concentration of the three molecules, and depends on the type of ingested beer, the nutritional context, the dose of ingested compound and the individual metabolization capacity of these polyphenols. Moreover, the doses studied here using topical administration or Matrigel injection with polyphenols are higher than reached dietary doses. Nonetheless, the pharmacological application of these promising compounds may also be of great interest. Serum concentrations of approximately 0.1 μM 8PN were observed after a single pharmacological dose of this polyphenol in post-menapausal women [Rad et al., 2006]. The concentrations employed may be achieved in specific tissues, by selective uptake, or repeated administration of 8PN [Brunelli et al., 2007], being possible for 8PN to be completely absorbed even at high concentrations in animals and probably also in humans [Pepper et al., 2004]. XN bioavailability is very low [Stevens and Page, 2004] and its metabolites were detected in plasma in the maximum concentration of 0.180 μM after oral administration to rats (50 mg/kg). In a very recent study, Bolca et al. [2010] found nM levels of these polyphenols in women serum after 5 days ingestion of these compounds. The most abundant polyphenols in human diet are not necessarily the most bioavailable ones or even the ones that have the most interesting effects. Even at very low concentrations, polyphenols may remain in blood for long enough to accumulate in target sites and to be able to exert their effects. The effects observed in rat wound-healing experiments led us to propose that further investigation on topic administration of these polyphenolic compounds should be done, as they might be a useful strategy for treating skin lesions, which involve often angiogenic and inflammatory impairment. The first anti-angiogenic drugs have only recently been approved for therapeutic use [Carmeliet, 2005] but due to emergent knowledge emphasizing the close partnership between inflammation and angiogenesis in several diseases, the development of therapeutic approaches against both angiogenesis and chronic inflammation may provide more effective therapies [Costa et al., 2007]. XN is able to inhibit tumor growth by targeting NFκB and Akt pathways and angiogenesis [Albini et al., 2006], has anti-inflammatory effects [Gerhauser, 2005b; Albini et al., 2006] and in vitro activity against Bcr-Abl+ cells, retaining cytotoxicity in

imatinib-resistant cells [Monteghirfo et al., 2008]. These characteristics render this molecule a promising drug. Food industry is also interested in the health benefits of these compounds, especially XN, and the production of a XN-enriched beer has gained interest in the brewing industry [Magalhaes et al., 2009]. According to the current study, IXN is also promising. Conversely, stimulation of angiogenesis can be very useful in the treatment of several pathological conditions such as tissue repair and ischemic conditions like ischemic stroke and heart and limb ischemia, rendering 8PN an interesting molecule.

In conclusion, these XN, IXN, and 8PN in vitro results outlined several angiogenic steps targeted by these polyphenols that were also confirmed by in vivo assays. Furthermore, the fact that these compounds also target SMC proliferation and migration, as revealed in this study, provides new evidence for the use of this agent in pathologies associated with altered SMC growth. Nevertheless, cellular and molecular mechanism need to be elucidated, since the mechanism of these polyphenols is still unclear.

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