The nicotine-derived 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK), present in tobacco smoke, is most likely involved in lung carcinogenesis in smokers. We demonstrated previously that non-steroidal anti-inflammatory drugs (NSAIDs) inhibit NNK-induced lung tumorigenesis, although the mechanism(s) is unknown. The present study demonstrates that, in U937 human macrophages, cyclooxygenase (COX)-1 and -2 are involved in the bioactivation of NNK to electrophilic mutagenic intermediates. We observed that acetylsalicylic acid and NS-398 decrease COX-dependent NNK activation in U937 cells by 66 and 37%, respectively. NSAIDs also decrease prostaglandin E2 (PGE2) synthesis, which is induced in a dose-dependent manner, reaching a 7-fold increase, in NNK-treated human U937 cells. We observed that NNK induces COX-1 expression and activates the nuclear factor-kB (NF-kB), in U937 cells. N-acetyl-l-cysteine and pyridil-inedithiocarbamate, two inhibitors of reactive oxygen species (ROS), inhibit NNK-induced PGE2 synthesis by 41 and 44%, respectively. These data suggest that ROS, generated during pulmonary metabolism of NNK could act as signal transduction messengers and activate NF-kB, which will subsequently induce COX-1 activity and increase PGE2 synthesis. These results reveal a novel aspect of tobacco carcinogenesis, and give us insight into the mechanisms of chemoprevention by NSAIDs. Accordingly, inhibition of NF-kB activation, leading to the inhibition of COX, offers a new approach in lung cancer prevention.

Introduction

The nicotine-derived N-nitrosamine, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK), present in tobacco smoke, is likely to be an important factor in the etiology of lung cancer (1). P450 monooxygenases activate NNK in human lungs (2). This activation, initiated by α-carbon hydroxylation, generates various electrophilic intermediates which alkylate specific sites of DNA (reviewed in ref. 3). We recently demonstrated that purified ovine cyclooxygenase (COX)-1 and -2 also activate NNK (4). A cyclooxygenase-dependent activation of NNK has never been demonstrated in humans.

The 5 year relative survival rate for lung cancer was estimated to be 12.3% in 1975 and 13.4% in 1990 (5). Considering the lack of progress in lung cancer therapy (5), chemoprevention offers a promising alternative to lung cancer control. Laboratory studies have demonstrated the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) against NNK-induced lung tumorigenesis (4,6). The anti-inflammatory activity is associated with an inhibition of COX metabolization of arachidonic acid (AA) to prostaglandin E2 (PGE2) (7). We recently demonstrated that these drugs inhibit NNK-induced PGE2 synthesis in tumor-bearing mice (4). COX-1 and -2 are encoded by separate genes (8). COX-1 expression appears to be tissue- and cell-specific although almost nothing is known about its mechanism of regulation. On the other hand, COX-2 is not expressed constitutively in the majority of the cell types, but can be induced through multiple signaling pathways (8). More importantly, the expression of COX-2 is significantly higher in human lung adenocarcinomas than in normal tissue (9). The mechanism(s) of inhibition of lung tumorigenesis by NSAIDs remains unknown, and the relationship between NNK metabolism and COX expression has not been established.

NNK bioactivation leads to the production of reactive oxygen species (ROS) (10). ROS are known to activate the nuclear factor-kB (NF-kB), which acts as a positive regulatory element of COX-2 expression (11,12). We hypothesize that cancer prevention by NSAIDs would involve an inhibition of NF-kB regulating COX expression, resulting in a reduction of NNK bioactivation. Here, we establish the relationship between NNK metabolism and COX expression in U937 human macrophages.

Materials and methods

Cell line

The human histiocytic lymphoma cell line U937 (ATCC) was routinely grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin at 37°C in a humidified 5% CO2 atmosphere (13). Cells in logarithm phase of growth were harvested, adjusted to 1×106 cells/ml in the presence of 10 nM PMA and incubated for 3 days in order to differentiate into adherent macrophages (14). Adherent cells were incubated in fresh complete RPMI 1640 medium in the absence of PMA for 6 h. Viability was >95% as determined by trypan-blue dye exclusion.

Abbreviations: AA, arachidonic acid; ASA, acetylsalicylic acid; COX, cyclooxygenase; LPS, lipopolysaccharides; NDMA, N-nitrosodimethylamine; NNK, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.
Bioactivation of NNK by PMA-differentiated U937 cells

Our first objective was to determine whether U937 human macrophages could metabolize NNK. We observed that PMA-differentiated U937 cells metabolize NNK (Table I, no. 1) along three pathways: activation initiated by α-carbon hydroxylation (10.0% representing 0.090 pmol/million cells/h); N-oxidation, considered a detoxication pathway (5.5%; 0.047 pmol/million cells/h), and carbonyl reduction (8.7%; 0.078 pmol/million cells/h), a reversible pathway producing 4-(methylnitrosamino)-1-(3-pyridyl)-1-butan-1-ol (NNAL) (3). PMA-differentiated cells, in which COX expression is further stimulated with TNF-α, showed activation (48.2%; 6.9 pmol/million cells/h) and N-oxidation (6.5%; 0.082 pmol/million cells/h) for NNK. Inhibitors specified in Table I were added to NNK-treated cells (50 μM) to determine the effects of PGE₂ on NNK metabolism. ASA (100 μM), a potent COX inhibitor, did not inhibit NNK metabolism, while SKF-525A (100 μM), a non-selective inhibitor of PGE₂ synthase, inhibited NNK metabolism by only 52% (P < 0.05) in unstimulated cells (no. 3), implicating enzymes other than COX-1 and COX-2. Inhibition of PGE₂ synthase did not change carbonyl reduction, yet it significantly reduced α-carbon hydroxylation. Under the incubation conditions, ASA (100 μM) hydrolyzed 90-95% of NNKOAc, while SKF-525A hydrolyzed 10.0% of NNKOAc to NNK and NNAL, consistent with the inhibition of PGE₂ synthesis. Inhibition of PGE₂ synthase did not change α-carbon hydroxylation, which was reduced by 64% in LPS-stimulated cells (no. 4), and by 52% in untreated cells (no. 3). ASA, a non-selective COX inhibitor, inhibited α-carbon hydroxylation by 64% in LPS-treated cells, indicating that COX-2 is a major target for NNK metabolism.

Results

In this study, we document the interaction between NNK metabolism and COX expression. Under all experimental conditions, cell viability was >95%.

Bioactivation of NNK by PMA-differentiated U937 cells

Our first objective was to determine whether U937 human macrophages could metabolize NNK. We observed that PMA-differentiated U937 cells metabolize NNK (Table I, no. 1) along three pathways: activation initiated by α-carbon hydroxylation (10.0% representing 0.090 pmol/million cells/h); N-oxidation, considered a detoxification pathway (5.5%; 0.047 pmol/million cells/h), and carbonyl reduction (8.7%; 0.078 pmol/million cells/h), a reversible pathway producing 4-(methylnitrosamino)-1-(3-pyridyl)-1-butan-1-ol (NNAL) (3). PMA-differentiated cells, in which COX expression is further stimulated with TNF-α, showed activation (48.2%; 6.9 pmol/million cells/h) and N-oxidation (6.5%; 0.082 pmol/million cells/h) for NNK. Inhibitors specified in Table I were added to NNK-treated cells (50 μM) to determine the effects of PGE₂ on NNK metabolism. ASA (100 μM), a potent COX inhibitor, did not inhibit NNK metabolism, while SKF-525A (100 μM), a non-selective inhibitor of PGE₂ synthase, inhibited NNK metabolism by only 52% (P < 0.05) in unstimulated cells (no. 3), implicating enzymes other than COX-1 and COX-2. Inhibition of PGE₂ synthase did not change carbonyl reduction, yet it significantly reduced α-carbon hydroxylation. Under the incubation conditions, ASA (100 μM) hydrolyzed 90-95% of NNKOAc, while SKF-525A hydrolyzed 10.0% of NNKOAc to NNK and NNAL, consistent with the inhibition of PGE₂ synthesis. Inhibition of PGE₂ synthase did not change α-carbon hydroxylation, which was reduced by 64% in LPS-treated cells (no. 4), and by 52% in untreated cells (no. 3). ASA, a non-selective COX inhibitor, inhibited α-carbon hydroxylation by 64% in LPS-treated cells, indicating that COX-2 is a major target for NNK metabolism.

Results

In this study, we document the interaction between NNK metabolism and COX expression. Under all experimental conditions, cell viability was >95%.
COX-1 and -2 inhibitor, was even more potent than NS-398, leading us to conclude that COX-1 would also metabolize NNK. The inhibition by A-79175, a specific 5-lipoxygenase inhibitor, demonstrates the involvement of a 5-lipoxygenase-dependent activation of NNK (no. 9) (23). Tepoxalin, a dual COX–5-lipoxygenases inhibitor (24,25) was the most effective of the drugs tested, reducing NNK bioactivation by 55%, thus confirming our conclusion above (no. 10). In order to determine whether NF-xB regulates COX expression in U937, we used CAPE or capsaicin, two inhibitors of NF-xB activation (26,27), which decreased NNK activation to the same extend as COX inhibitors (Table I, nos 7 and 8). Furthermore, we demonstrated by western blotting that CAPE and capsaicin inhibited COX-2 expression in LPS-treated cells (Figure 1).

**NNK-induced metabolization of exogenous AA in U937**

The extent of exogenous [3H]AA metabolism, reflected by the level of all five major and stable eicosanoids: 6Keto-PGF$_{1alpha}$, TXB$_2$, PGF$_{2alpha}$, PGE$_2$ and PGD$_2$, was increased by NNK treatment. LPS (100 ng, 4 h), used as positive control, doubled eicosanoid synthesis from [3H]AA (data not shown). As shown in Figure 2, eicosanoid synthesis was statistically higher in NNK (250 and 500 μM)-treated cells.

**Induction of COX-1 expression by NNK**

The increase in AA metabolism following NNK exposure suggests that COX-1 and/or COX-2 protein were induced. COX-1 protein was detected in PMA-differentiated U937 cells (Figure 3a). Surprisingly, immunoblot analysis revealed that NNK increased the amount of protein after an 18 h incubation (lanes 5 and 6, respectively). We observed that COX-2 is not constitutively expressed in U937 (lane 1) but can be induced by LPS (10 μg/ml, 6 h) (lane 2). Cells treated with NNK from 2 to 18 h, did not express COX-2 (lane 3, 2 h; lane 4, 6 h; lane 5, 12 h; lane 6, 18 h). Western blot analysis shows the effect of LPS as control (10 μg/ml, 4 h) and NNK (1 mM) on degradation of IκB-α in PMA-differentiated U937 cells. Lane 1 represents the basal level of IκB-α in unstimulated cells. After LPS treatment, used as control (50 μg/ml, 2 h), IκB-α band was undetected in the cytosolic fraction. Lanes 3–6, IκB-α degradation was maximal after 2 h of NNK treatment (1 mM) and the band density progressively increased after 4 h (lane 4), 6 h (lane 5) and 18 h (lane 6) without reaching constitutive level. Actin expression levels showed equal amounts of protein in all lanes. The data shown are from a single experiment and are representative of three separate experiments.

**Mechanism(s) of COX-1 induction by NNK**

To elucidate the mechanism(s) of induction of COX-1 by NNK, we measured the level of the major AA metabolite, PGE$_2$, secreted by U937 cells. We observed a dose-dependent induction of PGE$_2$ synthesis by NNK ($r^2 = 0.99$) (Figure 5a).
Fig. 4. Increase of COX-1 mRNA:28S rRNA ratio in NNK-treated U937 cells. Densitometry levels are expressed in arbitrary units. The figure represents duplicates (A and B).

Fig. 5. Modulation of PGE2 released by PMA-differentiated U937 cells. (a) Levels of PGE2 released after an 18 h incubation with NNK (□), NDMA (○), NDMAOAc (■) or NNKOAc (●). The r²-values for NNK and NDMA are 0.99 and 0.96, respectively. (b) Various inhibitors were added to NNK (500 µM) in the culture medium and PGE2 released was measured after an 18 h incubation period. Data are expressed as the percentage increase from unstimulated cells and are the means of two determinations. SE < 10%.

Used as control, for COX-2 induction, LPS (100 ng, 6 h) increased PGE2 synthesis 7-fold (data not shown). Another nitrosamine requiring bioactivation, NDMA, increased PGE2 synthesis. In contrast, NNKOAc and NDMAOAc, which produce selectively alkylating intermediates, did not induce PGE2 synthesis (Figure 5a) eliminating the hypothesis of the alkylating intermediate’s involvement in COX-1 induction. To verify if ROS are implicated in COX-1 induction, we added the free radical scavengers, N-acetyl-L-cysteine or pyrrolidine-dithiocarbamate, to NNK-treated cells and observed a partial inhibition of PGE2 synthesis (Figure 5b).

Absence of COX-2 induction by NNK

As expected, COX-2 protein was not observed in unstimulated cells (Figure 1). The addition of 1 mM NNK did not induce COX-2 expression during a 2–18 h exposition period (Figure 3b). In contrast to NNK, a 4 h LPS treatment had no effect on the level of COX-1 but induced COX-2. This suggests differences in the mechanisms of COX induction by LPS and NNK. To confirm the absence of COX-2 induction, we added NS-398 to NNK treated cells and measured the level of PGE2. As expected, this specific COX-2 inhibitor did not inhibit NNK-induced PGE2 synthesis (Figure 5b), whereas ASA, an inhibitor of both COX-1 and -2, decreased PGE2 level.

Inhibition of IκB-α by NNK

To support the hypothesis suggesting the involvement of NF-κB activation in NNK-induced COX expression, we measured the level of IκB-α. In resting cells, NF-κB is non-covalently associated with cytoplasmic inhibitory proteins such as IκB-α (reviewed in refs 28,29). Activation of NF-κB induces a cascade of events leading to the phosphorylation of IκB-α and its subsequent proteolytic degradation (28,29). IκB-α degradation, visualized by western blotting, correlates with NF-κB translocation to the nucleus, as measured by nuclear shift assay (28,29). In this study, a basal level of IκB-α was detected in the cytosolic fraction of unstimulated cells, whereas after NNK challenge, IκB-α was almost completely degraded (Figure 3c). We concluded that NNK activates NF-κB in human macrophages.

Discussion

NNK is a pro-carcinogen which is enzymatically activated into highly potent inducers of lung carcinogenesis (3). Average intake of NNK in smokers is 28 nmol per day or 410 µmol in 40 years (30). In this study, high concentrations of NNK had to be used to measure the effects of this carcinogen in human cells. Even if high doses of NNK were required, low concentrations of reactive metabolite intermediates were produced. Furthermore, cells were exposed to NNK for only 18 h, whereas smokers are exposed to this carcinogen for years. Here, we observed that NNK is bioactivated by COX-1 and -2 in human U937 macrophages, in addition to P450 monooxygenases and lipoxygenases. This is the first observation of a cyclooxygenase-dependent pathway of NNK bioactivation in human cells. We also observed that NNK induces prostaglandin synthesis in U937 human macrophages. Quite unexpectedly, NNK induces COX-1 expression but has no effect on COX-2 protein level. We also demonstrate, for the first time, that NNK activates NF-κB in macrophages and we
Fig. 6. Pathway of COX-1 induction by NNK in U937 macrophages. P450 monoxygenases, COX-1 and -2 and lipoxygenases activate NNK to reactive electrophilic intermediates (E\(^+\)) and ROS, causing DNA damage (3,4, 22). The addition of NNKOAc, an analogue of NNK which forms the same pyridyloxobutylating intermediate, to U937 will also generate DNA damage, but still not induce COX-1 expression. ROS produced during NNK bioactivation, induce PGE\(_2\) synthesis via an activation of NF-κB.

Previous human bioactivation studies of NNK have been restricted to whole lung tissues or microsomal preparations, rather than involving specific cell populations. These approaches cannot provide characteristics of specific pulmonary cell populations. In these systems, P450 monoxygenases and lipoxygenases have been shown to metabolize NNK (2,31). We demonstrated previously that purified ovine COX-1 and -2 activate NNK (4). Because they represent a specific human lung cell population and express COX isoenzymes, U937 macrophages were selected for this study (15). Furthermore, macrophages are indirectly implicated in carcinogenesis; they can serve as both positive and negative mediators of the immune system, and may contribute up to half of a lung tumor’s mass (reviewed in ref. 32). Here, we show for the first time that human U937 macrophages metabolize NNK. This suggests that alkylating intermediates produced during NNK activation by macrophages followed by migration to target pulmonary cells could generate migrations in these cells and initiate lung tumor development. In U937 cells, NNK bioactivation involves more than the P450 monoxygenases. Smith et al. (2) concluded that P450 monoxygenases mediate <50% of the total NNK metabolism in human lungs. PMA-differentiated U937 cells express COX-1 and -2 mRNA and corresponding proteins (33). Indeed, a significant finding of our study was that COX-1 and -2 metabolize NNK (Table I). We observed that 14.9% of the total amount of NNK was metabolized by purified ovine COX-1 and -2, and that anti-COX inhibited NNK activation in mouse lung (4). Our results are in line with a study showing that NNK is bioactivated by human lung cells, either as freshly un-separated cell digest, alveolar type II cells or alveolar macrophages (35).

We observed similar inhibition of NNK metabolism by blocking either COX activity or NF-κB activation. Interestingly, NF-κB is a positive regulatory element of the COX-2 response (11,12). Taken together, these data suggest a COX-dependent pathway of NNK metabolism in human lung which correlates with NF-κB activation. COX inhibitors, such as NSAIDs, prevent lung tumorigenesis by a still unknown mechanism of action (4,6). Our data suggest that NSAIDs, which prevent the activation of NNK to mutagenic electrophilic intermediates, act as anti-initiating agents in lung carcinogenesis.

We observed previously that NNK treatment increases plasma PGE\(_2\) levels in A/J mice (4). This observation led to the hypothesis that NNK could induce AA metabolism. Both COX isoenzymes metabolize AA to PGH\(_2\), which is then converted by specific enzymes, i.e. PGE synthase, PGI synthase, TX synthase, PGF\(_\alpha\) synthase and PGD synthase, to a variety of eicosanoids including PGE\(_2\) (36). In this study, we demonstrated that NNK induces both exogenous and endogenous AA metabolism in U937 cells, reflecting an induction of COX activity.

While human COX-1 and -2 metabolize NNK, the levels of COX-1 mRNA and protein are also induced by NNK metabolization, showing complex and multiple interactions regulating COX expression. NNK induces exogenous AA metabolism to eicosanoids (Figure 2), in parallel with an elevation of COX-1 protein. While an induction of COX-2 protein is commonly observed, an elevation of COX-1 in U937 was quite unexpected (8). Although not common, COX-1 induction has previously been observed in human breast tumors, in gastric mucosa from Helicobacter pylori-infected patients and in monocytoid cells treated with phorbol ester (37–39). The COX-1 gene is generally considered to be a non-inducible housekeeping gene, but details on the regulation of COX-1 expression are unknown. Nevertheless, Jims et al. (40) demonstrated that transcriptional activity of the COX-1 gene is significantly increased by interleukin-1β and tumor growth factor-β in prostate cancer cells. Furthermore, Wölfe et al. (41) recently observed that a 7 week treatment with the tumor promoter 2,3,7,8-tetrachlorodibenzo-p-dioxin induced the expression of COX-1 in mouse fibroblasts, up to 2-fold. Our observations further suggest that the mechanism of COX induction by NNK and LPS are different. LPS, a well known COX-2 inducer, does not affect COX-1 expression in U937 cells (Figure 3a) (15). Our data confirm that prevention of NNK-induced carcinogenesis by NSAIDs involves the inhibition of COX-1 and/or -2. PGs, such as PGE\(_2\), have multifunctional roles in controlling growth, metastasis and the host immune function (42).

COX-2 is not constitutively expressed in U937 cells, and the expression of this enzyme by NNK was not observed under our experimental conditions. The absence of induction by NNK in U937 human macrophages does not diminish the importance of COX-2 in carcinogenesis. El-Bayoumy et al. (43) demonstrated that NNK increasingly induces levels of COX-2 expression with progressive stages of lung tumorigenesis in rats. This suggests that COX-2 could be expressed at a later stage of carcinogenesis. In fact, Olivo et al. (44) reported that COX-2 expression is a further event to aberrant crypt foci formation, which are preneoplastic lesions in colon cancer. Furthermore, COX-2 was shown to be the constitutively expressed isoform in cultured human lung epithelial cells, and...
was induced in human lung adenocarcinomas, which are frequently observed in smokers (1,9,30). Since COX-1 and -2 expression varies widely among cells (8), it is possible that COX-2 is induced in other lung cell populations.

NF-κB regulates the protein transcripts of COX-2, cytokines, chemokines and cell adhesion molecules, which together play an important role in the immune and inflammatory response (28). In this study, we observed that NNK induces NF-κB activation in U937 cells. The induction of NF-κB by NNK, is delayed compared with more common activators, such as LPS (Figure 3c). This delay could be related to a slow bioactivation of NNK in U937 cells. Regulation of the COX-2 gene involves a complex array of regulatory factors including NF-κB (8). Our results suggest that NF-κB activation is not sufficient for COX-2 induction in U937. In contrast to COX-2, the regulation of the human COX-1 gene has been poorly documented. However, Kraemer et al. (45) observed three putative AP-1 binding sites on the murine COX-1 gene, suggesting a possible regulation of the COX-1 gene by nuclear factors.

To clarify the mechanism(s) of induction of COX-1 by NNK, we selected precursors of alkylating intermediates generated by specific pathways of NNK activation. We observed a dose-dependent induction of PGE2 synthesis by NNK. We extended this study to NDMA, another nitrosamine requiring bioactivation for carcinogenic activity. Like NNK, NDMA increases PGE2 synthesis. NNK is metabolized to methylation and pyridylidylobutylating intermediates which are strongly reactive electrophile intermediates implicated in lung carcinogenesis (3). We first focused our attention on those alkylating intermediates generated from NNK and NDMA, as possible inducers of PGE2 synthesis. We used NNKOAc and NDMAOAc, which are precursors of these alkylating intermediates. The hydrolysis of NNKOAc and NDMAOAc by esterases does not involve P450 monooxygenases and produces pyridylidylobutylating or methylating intermediates, exclusively and respectively (46). Surprisingly, these two precursors did not induce PGE2 synthesis eliminating the hypothesis of the alkylating intermediates involvement in COX-1 induction. This led us to propose a second mechanism of induction: ROS produced during NNK bioactivation (10) would induce PGE2 synthesis via an activation of NF-κB (Figure 6). In this study, we observed that ROS regulate NF-κB activation by NNK, although the free radical scavengers, N-acetyl-L-cysteine and pyrrolidinedithiocarbamate, added to NNK-treated cells did not completely inhibit PGE2 synthesis, suggesting that ROS are not the only factors regulating NF-κB activation by NNK. Tumor necrosis factor-α synthesis preceded NF-κB activation in U937, suggesting that this cytokine regulates NF-κB activation (47). We observed previously that NNK induces TNF-α in U937 (N.Rioux and A.Castonguay, manuscript submitted for publication). Janssen-Heininger et al. (48) recently observed that oxidants, such as ROS, and TNF cooperate to activate NF-κB. Taken together, these data suggest that TNF-α induction and ROS generated during NNK activation could, in cooperation, activate NF-κB, leading to COX-1 induction.

Our data suggest that ROS, generated during NNK metabolism (10), induce COX-1 expression possibly via NF-κB activation, as illustrated in Figure 6. Our current approach leads to a better understanding of the mechanism of action of NSAIDs in lung cancer prevention; this understanding is crucial in the development of preventive clinical trials. Inhibition of cyclooxygenase and/or NF-κB activation is a promising approach to lung cancer prevention.

Acknowledgements

We are grateful to Alain Tremblay and Dr Mare-Eduard Mirault for technical assistance with northern blotting. We also thank Abbott Laboratories and R.W. Johnson Pharmaceuticals for gifts of A-79175 and tepoxalin, respectively. We thank Jean-François Cloutier for the synthesis of NNKOAc and José Lamoureux for technical advice on immunoblotting. This work was supported by a grant from Cancer Research Society, Canada.

References


Received March 13, 2000; revised June 6, 2000; accepted June 14, 2000