Aspirin acetylates multiple cellular proteins in HCT-116 colon cancer cells: Identification of novel targets

SRINIVASAN MARIMUTHU[1], RAGHAVENDER S.V. CHIVUKULA[1], LLOYD F. ALFONSO[2], MAJID MORIDANI[3], FRED K. HAGEN[4] and G. JAYARAMA BHAT[1]

[1]Department of Biomedical Sciences, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, TX 79106; [2]D'Youville College School of Pharmacy, 320 Porter Avenue, Buffalo, NY 14201; [3]Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, TX 79106; [4]Proteomics Center, Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642, USA

Received May 2, 2011; Accepted June 10, 2011

DOI: 10.3892/ijo.2011.1113

Abstract. Epidemiological and clinical observations provide consistent evidence that regular intake of aspirin may effectively inhibit the occurrence of epithelial tumors; however, the molecular mechanisms are not completely understood. In the present study, we determined the ability of aspirin to acetylate and post-translationally modify cellular proteins in HCT-116 human colon cancer cells to understand the potential mechanisms by which it may exerts anti-cancer effects. Using anti-acetyl lysine antibodies, here we demonstrate that aspirin causes the acetylation of multiple proteins whose molecular weight ranged from 20 to 200 kDa. The identity of these proteins was determined, using immuno-affinity purification, mass spectrometry and immunoblotting. A total of 33 cellular proteins were potential targets of aspirin-mediated acetylation, while 16 were identified as common to both the control and aspirin-treated samples. These include enzymes of glycolytic pathway, cytoskeleton proteins, histones, ribosomal and mitochondrial proteins. The glycolytic enzymes which were identified include aldolase, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvate kinase M2, and lactate dehydrogenase A and B chains. Immunoblotting experiment showed that aspirin also acetylated glucose-6-phosphate dehydrogenase and transketolase, both enzymes of pentose phosphate pathway involved in ribonucleotide biosynthesis. *In vitro* assays of these enzymes revealed that aspirin did not affect pyruvate kinase and lactate dehydrogenase activity; however, it decreased glucose 6 phosphate dehydrogenase activity. Similar results were also observed in HT-29 human colon cancer cells. Selective inhibition of glucose-6-phosphate dehydrogenase may represent an important mechanism by which aspirin may exert its anti-cancer effects through inhibition of ribonucleotide synthesis.

Correspondence to: Dr G. Jayarama Bhat, Department of Biomedical Sciences, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, TX 79106, USA
E-mail: jayarama.gunaje@ttuhsc.edu

Key words: aspirin, acetylation, metabolic enzymes, glycolytic pathway, pentose phosphate pathway, anti-cancer effect

Introduction

Aspirin (acetylsalicylic acid), which is well known for its analgesic, anti-inflammatory and cardioprotective effects, also reduces the incidence of the epithelial cancers (1,2). Epidemiological studies carried over the past decade showed that regular intake of aspirin can decrease the incidence of cancer of the esophagus by 73, stomach 62, and colon by 63%. Cancer risk reduction was also observed in other organs such as breast (39%), lung (36%) and prostate tissue (39%) (3). *In vitro* studies have shown that aspirin is effective in inhibiting cell proliferation in a variety of cancer cell lines (4,5). In addition, it has been shown that regular aspirin use after the diagnosis of colorectal cancer lowers the risk and increases the overall patient survival (6). However, the molecular mechanisms leading to its anti-cancer effects are not clearly understood.

Aspirin consists of acetyl and salicylate moieties, both of which have their own distinct targets. While the salicylate group has been implicated in the anti-inflammatory properties of aspirin via the inhibition of NF-κB pathway (7), the acetyl group causes the inactivation of cyclooxygenases (COX) through acetylation of serine residues (8,9). Cyclooxygenases exists mainly in two forms: COX1 and COX2. Several mechanisms have been proposed to explain aspirin's chemopreventive properties. One widely accepted hypothesis is that aspirin decreases the cancer incidence by inhibiting COX2, as this enzyme is progressively over-expressed during the development of human colorectal cancers, and its disruption inhibits polyp formation in mice (10). However, other potential anti-cancer mechanisms, unrelated to COX also have been proposed (5,11). These include inhibition of NF-κB (7,12), induction of apoptosis by activation of p38 kinases (13), and catabolism of polyamines (14).

While aspirin's ability to acetylate and inhibit COX enzyme activity is well known (8,9), limited information is available as to whether it can acetylate other proteins and alter their functions (15,16). We recently demonstrated in MDA-MB-231 human breast cancer cells that one of the acetylation targets of aspirin is the tumor suppressor protein, p53 (17). In that report, we showed that aspirin acetylated lysine 382 (K382) on p53 and induced the expression of its target genes $p21^{CIP1}$ and Bax (17). Acetylation of p53 by aspirin was also observed in HCT-116 and HT-29 human colon cancer

cell lines (unpublished data). In another report, we showed that aspirin acetylated multiple cellular proteins in normal liver cells (18). Based on these observations, we hypothesized that exposure of other cell types to aspirin may cause acetylation of proteins, and identification of these targets would provide insight into the novel mechanisms on its anti-cancer effects.

Since significant cancer risk reduction is observed in colon tissue of patients who regularly take aspirin, we utilized human HCT-116 colon cancer cells in this study. We determined the pattern of aspirin induced acetylation of proteins using anti-acetyl lysine antibody specific for the recognition of acetylated lysine residues. The identity of the proteins was established by mass spectrometry and immunoblotting. Here, we demonstrate that aspirin exposure of cells caused acetylation of cytoskeletal proteins, histones, heatshock proteins, glycolytic pathway and pentose phosphate pathway enzymes. The enzymes identified include aldolase, glyceraldehyde 3 phosphate dehydrogenase (GAPDH), phosphoglyceromutase (PGM), enolase, pyruvate kinase isoform type M2 (PKM2), and lactate dehydrogenase (LDH-A and LDH-B), all of which are glycolytic enzymes; and glucose 6 phosphate dehydrogenase (G6PD) and transketolase, in the pentose phosphate pathway (PPP). Enzyme assays showed that despite acetylation, PKM2 and LDH enzyme activities were unaffected; in contrast, G6PD activity was inhibited with increasing concentrations of aspirin. Since G6PD regulates ribonucletide biosynthesis, its targeted inhibition by aspirin may represent an important mechanism by which it exerts its anti-cancer effects.

Materials and methods

Materials. Cell culture reagents were purchased from Invitrogen. Aspirin was obtained from Sigma. Anti-acetyl-lysine antibody was purchased from Immunechem Inc. Anti-pyruvate kinase M2 antibody, anti-phospho PKM2 antibody, anti-LDH-A antibody and anti-β-actin antibody were obtained from Cell Signaling Technology, Inc. Anti-enolase antibody, LDH-B antibody, anti-PGM antibody, anti-G6PD antibody, anti-transketolase antibody and anti-GAPDH antibody were obtained from Santa Cruz Biotechnology. All other chemicals were either from Sigma or Fisher.

Cell culture. HCT-116 and HT-29 cells were obtained from American Type Culture Collection (ATCC). Cells were grown for 12-24 h before the addition of aspirin for indicated times.

Preparation of cell lysate and immunoprecipitation. For immunoblotting experiments, cells were treated with aspirin for the indicated time and washed with phosphate buffered saline (PBS). Cells were scraped in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15% glycerol, 1% Triton-X-100, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Proteins (200 μg) were immunoprecipitated with anti-acetyl lysine antibody conjugated to agarose overnight and washed 3 times with lysis buffer. The proteins bound to agarose were eluted with 70 μl of triethylamine buffer (50 mM triethylamine, 150 mM NaCl) and neutralized with 12 μl of 2 M Tris (pH 6.8). The samples were then loaded onto a polyacrylamide gel, immunoblotted with respective primary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) Western blotting system according to the manufacturer's instructions (Pierce).

Sample preparation for mass spectrometry analysis, using LC-MS/MS. For mass spectrometry studies, 200 μg of cell lysates was immunoprecipitated using anti-acetyl lysine antibody conjugated agarose as described above. The immunoprecipitation (IP) resin was washed initially twice with lysis buffer, then once with detergent-free lysis buffer, and finally a last wash with 50 mM ammonium bicarbonate. The bound proteins were eluted, reduced, alkylated, and digested with either trypsin, or chymotrypsin using the methods described below. The peptide digests were subjected to LC-MS/MS analysis to identify peptide sequence matches.

Proteolytic digestion. Because total protein staining with Coomassie blue dye did not detect protein in the standard immunoprecipitate, the sample was scaled up, by conducting 20 identical immunoprecipitations. Proteins bound to anti-acetyl-lysine antibody resin were eluted at low pH elution, using an acidic volatile buffer. For the 20 vials, one 150 μl volume of a volatile acid buffer (VAB: 500 mM acetic acid, 20 mM ammonium acetate, pH 3.5) was added to the first tube and then sequentially transferred from one tube to the next, to elute the bound proteins from all 20 tubes. The eluant from the 20th tube was transferred into a LoBind tube containing 450 μl of 50 mM ammonium bicarbonate, to immediately neutralize the sample. A total of three acid treatments were performed and combined into the same tube. The sample was lyophilized and resuspended in 20 μl of 50 mM ammonium bicarbonate. To digest the proteins, 188 ng trypsin was added for an overnight digestion at 37˚C. The sample was reduced with 2 mM dithiothreitol (DTT) for 30 min, with shaking at 60˚C, and alkylated with 10 mM iodoacetamide (IAA) for 30 min in the dark at room temperature (RT). Finally, the alkylation reaction was quenched with 10 mM cysteine for 30 min at RT in the dark. A second aliquot of trypsin was added for 3 h to completely digest the sample. After digestion was complete, samples were lyophilized in a speedvac and resuspended in 5% acetonitrile, 0.05% formic acid and immediately loaded on a reverse phase nano-spray tip for LC-MS/MS analysis.

Mass spectrometry, using LC-MS/MS. Peptide digest (15%) was loaded on a Magic C18 AQ (Michrom) nanospray tip, packed to 5 cm. This tip is loaded, using a pressure bomb, and washed after installation on the HPLC of a Thermo LTQ mass spectrometer with 5% methanol, 0.1% formic acid, for 10 min with a flow rate of 600 nl/min (about 10 column volumes = 6.6 μl). The LTQ ion trap mass spectrometer was equipped with nano-electrospray ionization source. The peptides were eluted and analyzed in an LC-MS/MS run, using a 5-15% methanol gradient >2.5 min, followed by a 15-60% methanol gradient for 67 min, a 60% methanol isocratic step of 4 min, ending with a 3-min 95% methanol step, with all solvents containing 0.1% formic acid. A full MS survey scan is performed every 3 sec and the top 7 peaks were selected to collect MS/MS fragmentation spectrum, which were acquired in the data-dependent mode. The MS and fragmentation spectrum data was used in a Mascot search of the whole human proteome, to identify peptides derived from the proteins bound to the anti-acetylated lysine antibody column. Mascot search

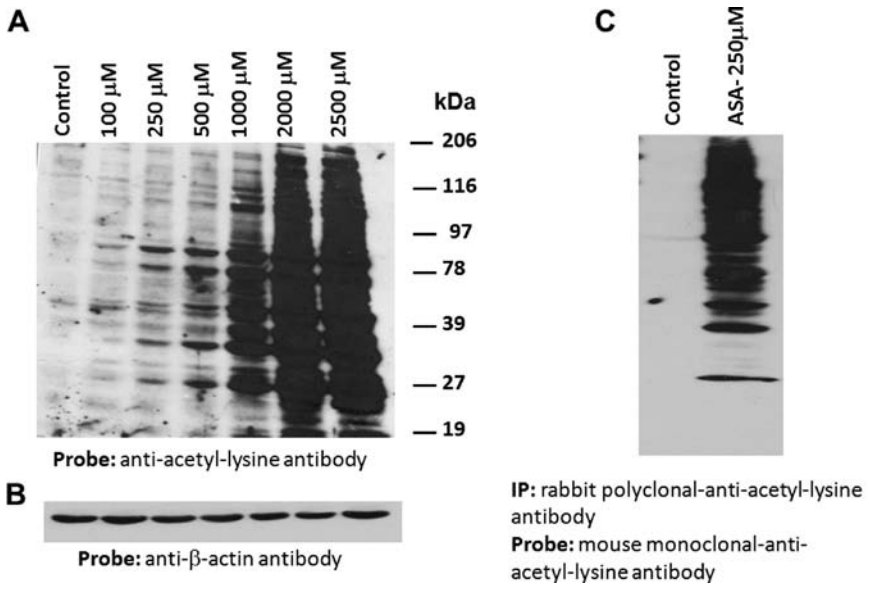

Figure 1. Aspirin acetylates multiple proteins in HCT-116 cells. (A) Concentration dependent protein acetylation by aspirin. Cells were grown in culture medium, left untreated or treated for 12 h with different concentrations of aspirin as indicated. Proteins were run on an 8% SDS-PAGE and immunoblotted with anti-acetyl lysine antibody. Protein bands were detected using enhanced chemiluminiscence (ECL). (B) The blot in (A) was stripped and reprobed with anti-β-actin antibody (loading control). (C) Immunoprecipitation of acetylated proteins by anti-acetyl lysine antibody. Cells were left untreated or treated with aspirin (250 μM) for 12 h, lysates prepared, 200 μg was immuprecipitated with rabbit anti-acetyl lysine antibody agarose conjugate. Protein was eluted from the resins and immunoblotted using mouse monoclonal anti-acetyl lysine antibody. Protein bands were detected using ECL. These experiments were repeated three times.

parameters include: precursor and fragment ion mass tolerance were set at 1.5 and 0.8 Da, respectively, and allowed for one C13 incorporation. Fixed carbamodomethyl-cysteine modification, variable methionine-oxidation and variable lysine acetylation were allowed in the Mascot search against the complete human proteome (NCBI 20110407). Because acetylation blocks trypsin cleavage, three missed trypsin cleavage sites were allowed in the search. The ion score/Expected cut-off score was set for 0.1 and peptides with an ions score greater than 50 were considered positive identification, if only one peptide was identified for a given protein.

PKM2, LDH and G6PD assays. Subconflent cells were treated with aspirin at different concentration; cells lysed using the assay buffer according to the manufacturer's instructions (BioVision Inc). The enzymatic assays for PKM2, LDH, G6PD were measured in 96-well plates. In PKM2 assay, phosphoenol pyruvate and adenosine diphosphate are catalyzed by pyruvate kinase to generate pyruvate and adenosine triphosphate. The generated pyruvate is oxidized by pyruvate oxidase to produce color which is measured at 570 nm. For LDH assay, LDH reduces NAD to NADH which then interacts with specific probe to produce a colour which is measured at 450 nm. For the G6PD assay, glucose 6 phosphate is oxidized with the generation of a product which is utilized to convert a nearly colorless probe to an intensely colored product with an absorbance at 450 nm.

Results

Aspirin acetylates multiple cellular proteins in human colon cancer cells. Recent reports in literature have demonstrated that acetylation of cellular proteins is a widespread phenomenon and this posttranslational modification can regulate protein functions (19-21). The array of proteins that undergo acetylation within the cells include transcription factors, histones, metabolic pathway enzymes and cytoskeletal proteins to name few. Since aspirin is known to acetylate cyclooxygenases (COX) through the non-enzymatic reaction, aminolysis (22), we hypothesized that aspirin's range of targets may also include other cellular proteins. To determine this, HCT-116 cells were treated with aspirin at different concentrations for 12 h, lysates prepared and immunoblotted with anti-acetyl lysine antibody. Fig. 1A demonstrates that very few acetylated proteins were detected in the untreated control; however, low amounts of aspirin acetylated proteins were detected at 100 μM concentration, but this increased significantly thereafter. The molecular weight of these proteins ranged from 20 to 200 kDa. Reprobing the blot with anti-β-actin antibody showed equal amounts of protein levels in all lanes (Fig. 1B; loading control). Aspirin-mediated acetylation was also observed in HT-29 cells with similar acetylation profiles (data not shown).

Identification of proteins by mass spectrometry. We then sought to determine the identity of acetylated proteins through mass spectrometry. Since the acetylation levels of proteins detected at 100 μM were low, 250 μM aspirin was used to treat cells to quantitatively recover the acetylated proteins in immunoprecipitation reactions. For this, cells were treated with aspirin at 250 μM for 12 h, proteins immunoprecipitated with anti-acetyl lysine antibody conjugated agarose. Multiple immunoprecipitations were carried out and pooled to ensure that enough proteins were collected for mass spectrometry. Fig. 1C demonstrates that polyclonal anti-acetyl lysine antibody successfully immunoprecipitated acetylated proteins in aspirin treated cells (lane 2). Mass spectrometry analysis identified a total of 16 proteins common between untreated control and aspirin treated samples, whereas 33 unique proteins were identified in aspirin treated samples (Tables I and II). Their reference accession numbers, function

Table I. Proteins in ASA-treated IP only.

SNO	Protein name	Acc. no	Protein function	Representative peptide sequence
1.	Pyruvate kinase	Gi:73535278	Metabolism	K.IYVDDGLISLQVK.Q
2.	Aldolase A	Gi:49456715	Metabolism	K.GILAADESTGSIAK.R
3.	Lactate dehydrogenase A chain isoform 1	Gi:62897717	Metabolism	K.LLIVSNPVDILTYVAWK.I
4.	L-lactate dehydrogenase B chain	Gi:49259209	Metabolism	R.VIGSGCNLDSAR.F
5.	Chain R, twinning in crystals of human skeletal muscle D-glyceraldehyde-3-phosphate dehydrogenase	Gi:230867	Metabolism	K.IISNASCTTNCLAPLAK.V
6.	Glutamic-oxaloacetic transaminase 2 R.FVTVQTISGTGALR.I mitochondrial	Gi:73486658	Metabolism	
7.	Phosphoglycerate mutase	Gi:67464262	Metabolism	R.VLIAAHGNSLR.G
8.	Full=putative elongation factor 1-α-like 3 protein	Gi:74746925	Protein synthesis	K.SGDAAIVDMVPGKPMCVESFSDYPPLGR.F
9.	60S acidic ribosomal protein P2	GI:4506671	Protein synthesis	K.NIEDVIAQGIGK.L
10.	Signal recognition particle 9 kDa protein isoform 2	Gi:4507217	Protein synthesis	M.PQYQTWEEFSR.A
11.	Elongation factor-2	Gi:4503483	Protein synthesis	R.ETVSEESNVLCLSK.S
12.	60S ribosomal protein L22 proprotein	Gi:4506613	Protein synthesis	K.AGNLGGGVVTIER.S
13.	Ribosomal protein L4 variant	Gi:62087534	Protein synthesis	K.AAAAAAALQAK.S
14.	Chaperonin 10-related protein	Gi:4504523	Protein folding	K.GKGGEIQPVSVK.V
15.	60 kDa heat shock protein, mitochondrial	Gi:77702086	Protein folding	K.TLNDELEIIEGMK.F
16.	90 kDa heat shock protein	Gi:6807647	Protein folding	R.GVVDSEDLPLNISR.E
17.	MTHSP75	Gi:292059	Protein folding	K.VQQTVQDLFGR.A
18.	Cofilin-1	Gi:73983054	Cytoskeleton	R.YALYDATYETK.E
19.	Cyclophilin	Gi:48145775	Cytoskeleton	K.TVDNFVALATGEK.G
20.	Annexin A2	Gi:73909156	Cytoskeleton	K.TPAQYDASELK.A
21.	Annexin A1	Gi:4502101	Cytoskeleton	K.TPAQFDADELR.A
22.	Metallothionein MT IIpg	Gi:90109444	Antioxidant	K.SCCSCCPVGCAK.C
23.	D-dopachrome decarboxylase	Gi:4699610	Inflammatory cytokine	R.FFPLESWQIGK.I
24.	UV excision repair protein RAD23 homolog B	Gi:4506387	DNA repair protein	K.NFVVVMVTKPK.A
25.	ATP synthetase subunit d. mitochondrial isoform a	Gi:5453559	Respiration	R.LAALPENPPAIDWAYYK.A

Table I. Continued.

SNO	Protein name	Acc. no	Protein function	Representative peptide sequence
26.	LIM protein	Gi:63989934	Cytoskeleton	R.IGDVVLSIDGINAQGMTHLEAQNK.I
27.	Similar to calgizzarin: similar to PID: q3115349	Gi:5032057	Cytoskeleton and anti-inflammatory	K.TEFLSFMNTELAAFTK.N
28.	Hornerin	Gi:57864582	Cornification	R.GPYESGSGHSSGLGHR.E
29.	Thioredoxin-dependent peroxide reductase	Gi:62896877	Antioxidant	K.HLSVNDLPVGR.S
30.	Hypothetical protein DKFZp762H157.1-human : ezrin protein	Gi:46249758	Cytoskeletal protein	K.SQEQLAAELAEYTAK.I
31.	thymosin β-10	Gi:339697	Actin binding	.DCFKKMADKPDMGEIASFDK.A
32.	thymosin β-4	Gi:10946578	Actin binding	M.SDKPDMAEIEKFDK.S
33.	Ribosomal protein S21	Gi:3088341	Protein synthesis	R.MGESDDSILR.L

Proteins identified through mass spectrometry which are unique to aspirin treated conditions. IP, immunoprecipitation; ASA, aspirin.

Table II. Proteins common to control and ASA-treated IP.

SNO	Protein name	Acc. no	Protein function	Representative peptide sequence
1.	Glyceraldehyde-3-phosphate dehydrogenase	Gi:7669492	Metabolism	R.VPTANVSVVDLTCR.L
2.	α-enolase	Gi:62897945	Metabolism	R.AAVPSGASTGIYEALELR.D
3.	Malate dehydrogenase precursor	Gi:93279232	Metabolism	K.VDFPQDQLTALTGR.I
4.	40S ribosomal protein S28	Gi:4506715	Protein synthesis	R.EGDVLTLLESER.E
5.	60S ribosomal protein L29	Gi:793843	Protein synthesis	K.AQAAAPASVPAQAPK.R
6.	Chain A. free acetyl-cypa trigonal form	Gi:291463382	Immunosuppressant	M.VNPTVFFDIAVDGEPLGR.V
7.	Profilin-1	Gi:5822002	Actin-binding protein	K.TFVNITPAEVGVLVGK.D
8.	Actin, α skeletal muscle	Gi:4885049	Cytoskeleton	K.SYELPDGQVITIGNER.F
9.	Histone H1b	Gi:51315727	Transcription regulators	K.ASGPPVSELITK.A
10.	Histone H4	Gi:51315727	Transcription regulators	K.TVTAMDVVYALK.R
11.	Peroxiredoxin-1	Gi:55959887	Anti-oxidant	K.QGGLGPMNIPLVSDPK.R
12.	Host cell factor	Gi:98986457	Cell cycle and transcriptional regulation	K.SPISVPGGSALISNLGK.V
13.	Macrophage migration inhibitory factor	Gi:5542325	Lymphokine involved in cell mediated immunity	R.SYSKLLCGLLAER.L
14.	Chain H, structure of the hirulog 3-thrombin complex and nature of The S' subsites of substrates and inhibitors	Gi:493792	Coagulation	K.SPQELLCGASLISDR.W
15.	Thymosin β-10	Gi:10863895	Actin binding	M.ADKPDMGEIASFDKAK.L
16.	hCG1993380, isoform CRA_a	Gi:119607608	Miscellaneous	R.RPSAAAKPSGHPPPGDFIALGSK.G

Proteins identified through mass spectrometry which are common between untreated and aspirin treated conditions. IP, immunoprecipitation; ASA, aspirin.

and a representative peptide identified are shown. Some of the notable proteins in this list include metabolic pathway enzymes such as aldolase, α-enolase, PGM, GAPDH, PKM2, LDH-A and B chains, glutamic-oxaloacetic transaminase 2 and malate dehydrogenase; cytoskeletal proteins such as Annexin A1 and A2; and DNA binding proteins such as histone H1b and histone H4. Definitive evidence for protein acetylation was achieved by identification of acetylated peptides. Mass spectrometry fragmentation spectrum analysis revealed 5 acetylated peptides from 5 aspirin-treated proteins and an additional 3 acetylated peptides from both the treated and untreated immunoprecipitated samples (Table III). The acetylated peptides map to 5 of 6 known native acetylation sites in thymolysin β-10, histone H4 and hCG1993380 isoform CRA, demonstrating that expected acetylation sites are successfully mapped, using LC-MS/MS method. Mass spectrometry identification of acetylated peptides specifically in the aspirin-treated sample, indicates that proteins are indeed modified by the treatment and are enriched by the immunoprecipitation protocol.

Confirmation of acetylation of glycolytic pathway enzymes by aspirin. Although mass spectrometry analysis identified many proteins as potential targets of aspirin, in this study, we focused initially on aspirin's ability to acetylate glycolytic enzymes. Because cancer cells exhibit increased rate of glycolysis and lactate production (23-25), we considered the possibility that aspirin acetylation of glycolytic enzymes might alter their function. Immunoblotting experiments were performed to further confirm the acetylation of these enzymes. In these experiments, we used aspirin at a concentration of 100 μM as this concentration has been demonstrated to be physiologically achievable on administration of an oral dose (1.2 g) (26). Cell lysates were prepared from untreated and aspirin-treated cells for 12 h, equal amounts of proteins immunoprecipitated with anti-acetyl lysine antibody agarose conjugate, proteins eluted and immunoblotted with either anti-aldolase antibody, or anti-GAPDH antibody, or anti-PGM antibody, or anti-enolase antibody, or anti-PKM2 antibody, or anti-LDH A and B antibodies. We observed that aspirin acetylated aldolase (Fig. 2A), GAPDH (Fig. 2C), PGM (Fig. 2E), enolase (Fig. 2G), PKM2 (Fig. 2I), LDH-A (Fig. 2K) and LDH-B (Fig. 2M) enzymes. Among these, enolase was constitutively acetylated and this was significantly increased following aspirin treatment (Fig. 2G). The amount of proteins between control and aspirin treated conditions remained similar suggesting that aspirin does not alter the levels of these enzymes; but causes their acetylation (Fig. 2B, D, F, H, J, L and N).

Aspirin acetylates PKM2 but does not alter phosphorylation at Y^{105}. PKM2 exists in two forms: the active tetrameric form; and the less active dimeric form (24). It was shown that oncoproteins such as fibroblast growth factor receptor type 1 can phosphorylate the tyrosine 105 (Y^{105}) on PKM2 and this leads to prevention of the formation of active tetrameric form and inhibition of enzyme activity (27). Since aspirin acetylates PKM2 (Fig. 2I), we determined whether this modification has any effect on the tyrosine phosphorylation at Y105 of the enzyme. For this, cells were left untreated or treated with aspirin (100 μM), equal amounts of proteins immunoblotted with anti-phospho PKM2 antibody. Fig. 3A demonstrates that aspirin treatment did not alter phosphorylation at Y105

Table III. Acetylated peptides from control and ASA-treated cells.

SNO	Protein name	Acc. no	Protein function	Occurrence	Acetylated* peptide sequence
1.	Histone H4	Gi:51315727	Transcription regulator	Both treated and control	K.GGK*GLGK*GGAK*R.H
2.	hCG1993380, isoform CRA_a	Gi:119607608	Miscellaneous	Both treated and control	R.RPSAAAK*PSGHPPPGDFIALGSK.G
3.	Thymosin β-10	Gi:10863895	Actin binding	Both treated and control	M.ADK*PDMGEIASFDK.A M.ADK*PDMGEIASFDK*AK.L
4.	Macrophage migration inhibitory factor	Gi:5542325	Lymphokine involved in cell mediated immunity	ASA-treated	R.SYSK*LLCGLLAER.L
5.	DKFZp762H157.1-ezrin protein	Gi:46249758	Cytoskeletal protein	ASA-treated	R.QAVDQIK*SQEQLAAELAEYTAK.I
6.	Thymosin β-4	Gi:10946578	Actin binding	ASA-treated	M.SDK*PDMAEIEK*FDK.S
7.	Chaperonin 10-related protein	Gi:4504523	Protein folding	ASA-treated	K.GK*GGEIQPVSVK.V
8.	Cyclophilin	Gi:48145775	Immunosuppressant	ASA-treated	K.IEVEKPFAIAK*E.-

Acetylated peptides identified from untreated and aspirin treated conditions. ASA, aspirin.

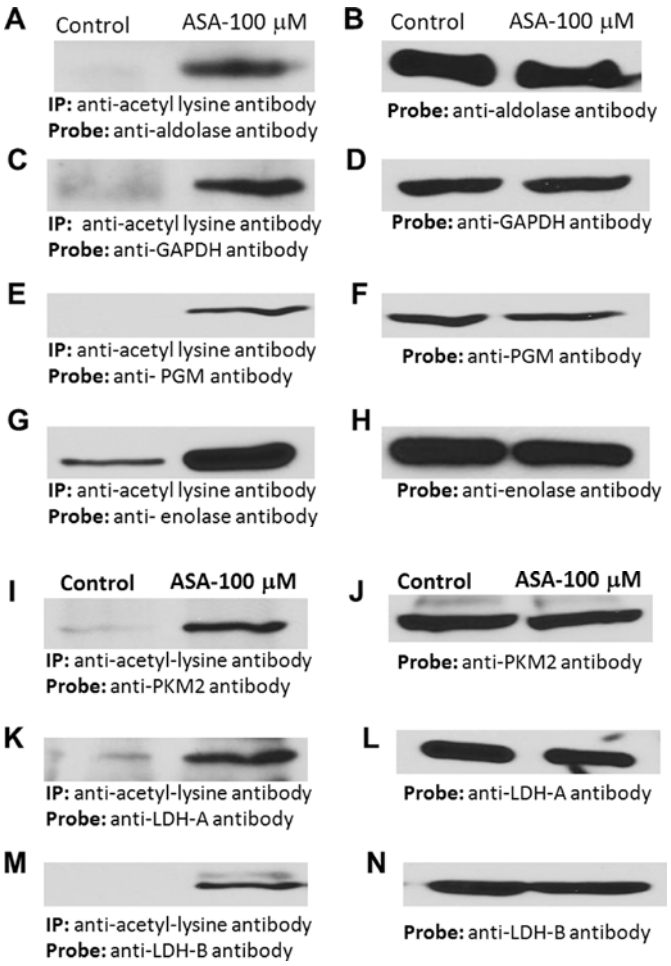

Figure 2. Aspirin acetylates glycolytic pathway enzymes. (A, C, E, G, I, K and M). Cells were left untreated or treated with aspirin at 100 μM for 12 h, lysates prepared, equal amounts of proteins (200 μg) immunoprecipitated with rabbit anti-acetyl lysine antibody agarose conjugate. Protein was eluted from the resins and immunoblotted using respective antibodies as indicated. (B, D, F, H, J, L and N), Twenty microgram of the samples representing A, C, E, G, I, K and M) were run on a SDS- PAGE (8 or 12%) and immunoblotted with respective antibodies. Protein bands were detected using ECL. These experiments were repeated three times.

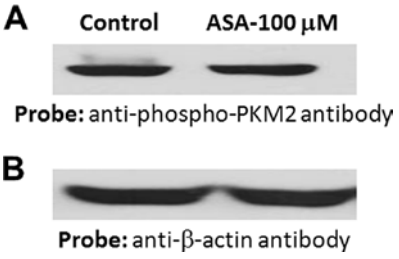

Figure 3. Aspirin does not alter PKM2 phosphorylation at Y^{105}. (A) Cells were left untreated or treated with aspirin at 100 μM for 12 h, lysates prepared, equal amounts of proteins (20 μg) loaded onto an 8% SDS-PAGE and immunoblotted with anti-phospho PKM2 antibody. (B) The blot in (A) was stripped and reprobed with anti-β-actin antibody. Protein bands were detected using ECL. These experiments were repeated four times.

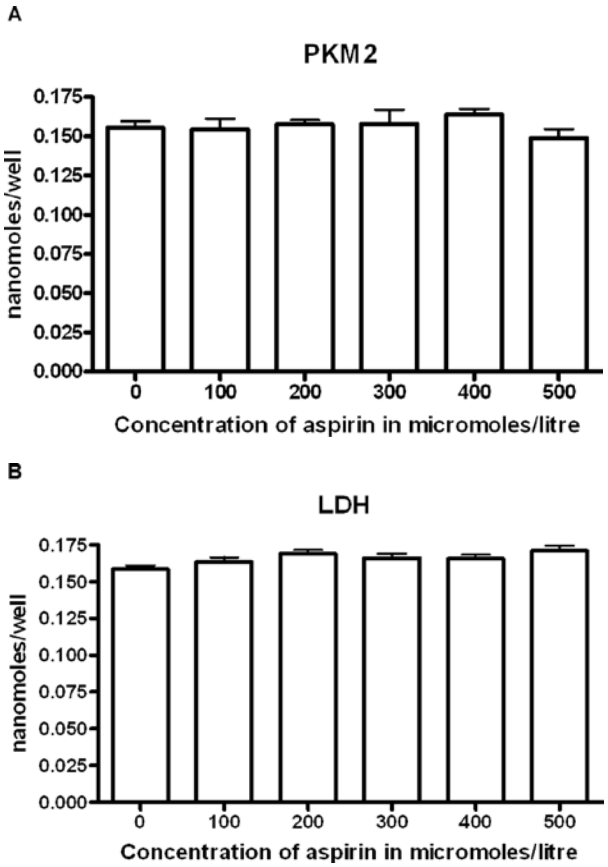

Figure 4. Aspirin does not alter the PKM2 and LDH enzyme activity. (A) Cells were seeded on 100 mm petridish overnight and treated with different concentrations of aspirin for 24 h. Cells ($2x10^6$) were homogenized in the assay buffer using a dounce homogenizer, lysates centrifuged and supernatant collected and protein estimated. For the PKM2 activity, 1 μg of the protein was used according to the protocol (Biovision Inc.). Following 30 min of incubation at room temperature and color development, the absorbance was measured at 570 nm. The pyruvate that is generated was expressed in nanomoles/well. (B) The samples were prepared as in A, 0.5 μg of the protein was used for the LDH assay according to the protocol (Biovision Inc.). Following 30 min of incubation at room temperature, the absorbance was measured at 450 nm. The NADH generated was expressed in nanomoles/well. Results are means ± SEM (n=3). The data were analyzed using one-way ANOVA followed by Bonferonni's-multiple comparison test and no statistically significant change was observed across different groups.

on PKM2 compared to untreated control. These samples contained equal amounts of β-actin protein (Fig. 3B, loading control).

PKM2 and LDH acetylation by aspirin does not alter their enzyme activity. PKM2 isoform of pyruvate kinase has been shown to be important for cancer metabolism and tumor growth (28). Similarly, lactate dehydrogenase A also has been implicated in tumor progression (29). Pyruvate kinase M2 and LDH are enzymes which respectively catalyze the conversion of phosphoenol pyruvate to pyruvate, and pyruvate to lactate. Since acetylation is known to alter biological activity in many proteins (19-21), we determined whether aspirin-mediated acetylation effects the activity of these two enzymes. For this, cells were untreated or treated with increasing concentrations of aspirin (100-500 μM); PKM2 and LDH enzyme activities were measured. We observed that aspirin treatment in HCT 116 cells had no effect on PKM2 (Fig. 4A) and LDH (Fig. 4B) enzyme activity over the range of concentrations tested.

Aspirin acetylates pentose phosphate pathway (PPP) enzymes glucose 6 phosphate dehydrogenase (G6PD) and transketolase. We considered the possibility that additional proteins may get

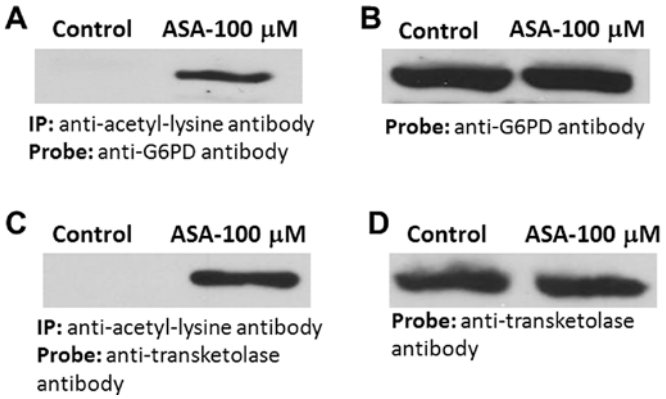

Figure 5. Aspirin acetylates G6PD and transketolase. (A and C) Cells were left untreated or treated with aspirin at 100 μM for 12 h, lysates prepared, 200 μg of proteins immunoprecipitated with rabbit anti-acetyl lysine antibody agarose conjugate. Protein was eluted from the resins and immunoblotted with anti-G6PD or anti-transketolase antibody. (B and D) Twenty microgram of the samples representing (A) and (C) was run on a 12% SDS-PAGE and immunoblotted with anti-G6PD or anti-transketolase antibody. Protein bands were detected using ECL. These experiments were repeated four times.

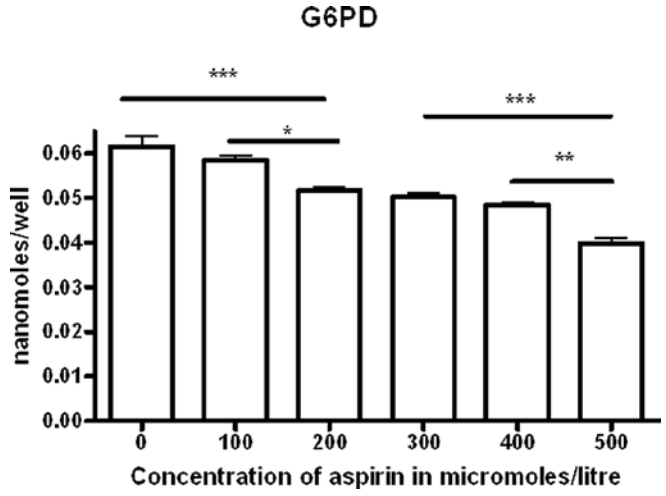

Figure 6. Aspirin inhibits G6PD enzyme activity. Cells were cultured and treated with aspirin as described in Fig. 5. One hundred μg of the protein was used for the G6PD assay according to the protocol (Biovision Inc.). The reaction mixture was incubated at 37˚C for 30 min, the absorbance measured at 450 nm. The NADH generated was expressed in nanomoles/well. Results are means ± SEM (n=3). The data were analysed using one-way ANOVA followed by Bonferonni's-multiple comparison test, giving p-value between each group, $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

acetylated with aspirin, but are below the limits of detection of mass spectrometry. Moreover, some proteins may go undetected as abundant non-acetylated peptides may compete with acetylated peptides for detection (30). To explore the possibility that additional metabolic proteins such as G6PD and transketolase become acetylated, but are difficult to detect via mass spectrometry, we enriched the acetylated proteins after 100 μM aspirin treatment by conducting an anti-acetyl lysine immunoprecipitation and probing with anti-G6PD and anti-transketolase antibodies. G6PD and transketolase respectively are two key enzymes in the oxidative and non-oxidative phase of PPP and play a role in nucleotide bio-synthesis (31,32). In Western blots, we observed that aspirin acetylated both G6PD (Fig. 5A) and transketolase (Fig. 5C), where as in the untreated control, acetylation of these enzymes were undetected. The cell lysates used for immunoprecipitation contained similar amounts of G6PD and transketolase protein levels suggesting that aspirin does not alter their expression levels (Fig. 5B and D). Similar results were obtained in HT-29 cells (data not shown).

Acetylation of G6PD is associated with decreased enzyme activity. To determine if acetylation of G6PD affects its enzyme activity, HCT-116 cells were untreated or treated with different concentrations of aspirin (100-500 μM), G6PD activity was measured. Fig. 6 demonstrates that aspirin progressively inhibited G6PD activity particularly beginning at 200 μM. Numerically, the percentage inhibition was found to be 7% at 100 μM, 17% at 200 μM, 20% at 300 μM, 23% at 400 μM and 35% at 500 μM. Similarly, treatment of HT-29 cells with aspirin also reduced G6PD activity in (data not shown). This suggests that acetylation of G6PD by aspirin causes a decrease in its enzyme activity.

Discussion

Aspirin's chemopreventive effects are increasingly being recognized; however, a plausible explanation for its anti-cancer effect is only beginning to be understood. We previously demonstrated in the human breast cancer cell line MDA-MB-231 that aspirin acetylates the tumor suppressor protein p53 at K382, and induces expression of its target genes (17). The present study was carried out to explore if aspirin acetylates other cellular proteins and modulate their function, which potentially could explain its anti-cancer effects. In this study, we demonstrate for the first time that aspirin, within the range of physiologically achievable concentration (100-300 μM) (33-35), acetylated multiple proteins at lysine residues in both HCT-116 and HT-29 colon cancer cells. Through mass spectrometry and immunoblotting, we identified several acetylation targets for aspirin. These include metabolic enzymes, cytoskeletal proteins, heat shock proteins, histones as well as some mitochondrial proteins. This is an important observation because, till date, very few proteins are known to be acetylated in cells exposed to aspirin (8,17,36). Thus, our finding extends the previously known list of proteins acetylated by aspirin suggesting that some of its unexplained therapeutic properties may occur through acetylation of proteins.

In this study, we identified a total of 49 proteins as potential targets for aspirin mediated acetylation using mass-spectrometry. Among these, few proteins that are identified may not be the true targets of aspirin, as unmodified proteins may co-precipitate with acetylated proteins by non-specific adsorption in immunoprecipitation reactions. Among the proteins that were identified as potential targets, we focused on the enzymes of glucose metabolism due to their importance in cancer (24). Rapidly growing cancer cells are known to have glycolytic rates that are up to 200 times higher than that of normal cells (23). This increased rate of glucose utilization has been attributed to mainly to the expression of PKM2 isoform in cancer cells (28), although some studies have implicated a role for hexokinase II in this process (37). Under normal conditions PKM2 is expressed in embryonic and rapidly growing cells; however, its expression is observed at elevated levels in cancer cells (24). Similarly, another report showed that LDH-A is important for tumor progression (29). In our study, six enzymes in the glycolytic pathway were

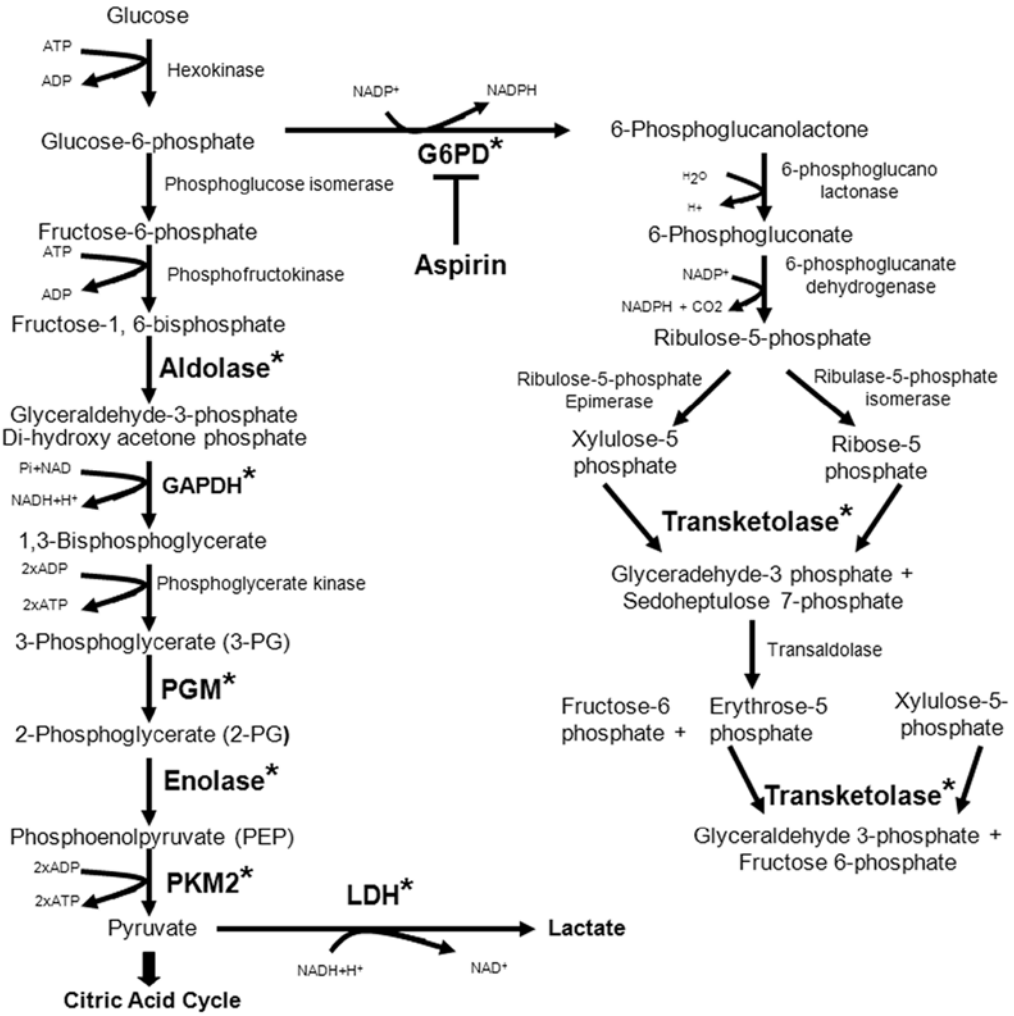

Figure 7. Model depicting the acetylation targets of aspirin on glycolysis/pentose phosphate pathway enzymes. Aspirin acetylates six enzymes in glycolysis and two enzymes in pentose phosphate pathway (shown in bold and with an asterisk). Note that enzyme inhibition is observed with G6PD.

found to be acetylated upon aspirin treatment. These include aldolase, GAPDH, PGM, enolase, PKM2, LDH-A and LDH-B. Among these, most abundant acetylation was observed for enolase by aspirin. Interestingly, enolase was constitutively acetylated although to a lower level and this is consistent with previous reports (38). Despite acetylation of PKM2, its activity remained unchanged upon aspirin treatment. Aspirin also did not alter PKM2 phosphorylation at Y^{105} which further supports the view that enzyme activity remains unaltered following aspirin treatment. Some reports show that PKM2 is differentially compartmentalized within the cells (24); however, it is not clear if aspirin-induced acetylation affects its compartmentalization. Similarly, LDH activity was also unaffected upon aspirin treatment. In immunoblotting experiments we did not see acetylation of hexokinase II by aspirin (data not shown) which suggests that some enzymes in glycolytic pathway are not the targets of aspirin. Our results suggest that aspirin post-translationally modifies a wide range of proteins with probably neutral impact on bulk protein function.

While the amount of protein concentrated in the immunoprecipitation reaction was low and posed some limits on detection by mass spectrometry, we identified G6PD and transketolase as the target of aspirin mediated acetylation by using immunoblotting experiments. This observation reflects the well-known fact that immunoblotting techniques are more sensitive detection methods than mass spectrometry, particularly for low abundance proteins. G6PD is the key regulatory enzyme in PPP and necessary for the synthesis of nucleotides and nucleic acids (39-41). Recent studies have demonstrated that G6PD activity is suppressed in cells containing wild-type p53 through a direct inhibition; whereas, it is not subjected to such negative regulation in cells containing mutant p53 (42). This has been implicated in the increased consumption of glucose in tumor cells containing mutant p53. Our previous reports show that aspirin has the ability to acetylate and activate both wild-type and mutant p53 (17). In this study, we observed that acetylation of G6PD by aspirin was associated with inhibition of its activity. This is a novel finding because the targeted inhibition of G6PD has been proposed as a strategy to inhibit nucleic acid synthesis in cancer therapy (43). Several studies have demonstrated the potential of inhibiting G6PD to reduce cell proliferation *in vitro* and *in vivo* (31,44,45). For example, the ability of the dehydroepiandrosterone to inhibit G6PD and to inhibit cancer cell proliferation has been documented (32,40,44). It is not clear at this stage if G6PD inhibition

observed in aspirin treated cells is due to an interaction between acetylated p53 and G6PD, or is the result of acetylation of G6PD itself. Our finding that aspirin inhibits G6PD suggests that inhibition of PPP could be one mechanism that contributes to the anti-cancer/anti-proliferative effects of aspirin.

In this study, we have not investigated the importance of the increased acetylation of other proteins that were identified through mass-spectrometry for their significance in aspirin's anti-cancer effects. These include, heat shock proteins, histones, ribosomal proteins and annexins whose modification could also play a role. For example, hyper-acetylation of HSP-90α has been shown to increase its association with MMP-2 leading to increased tumor cell invasiveness (46). One of the proteins that was identified with significant Mascot score in mass-spectrometry is HSP90α. We have confirmed its acetylation in immunoblotting experiments (data not shown). Annexin, a cytosketal protein, is involved in diverse cellular processes such as epithelial cell motility and cell matrix interactions (47). It has been shown that histone acetylation has the capacity to destabilize the chromatin polymer through charge neutralization of the basic lysine residues leading to changes in the chromatin structure and transcription of genes (30). Moreover, inhibitors of histone deacetylases have been used in chemotherapy (48). Chemical acetylation by aspirin can play a role in shifting the equilibrium of the enzymatic acetylation/deacetylation process. It remains to be determined how acetylation of these proteins could affect their functions and whether these contribute to aspirin's anti-cancer effects.

Protein lysine acetylation over the past decade has emerged as a key post-translational modification in cellular regulation of both histone and transcription factors (49). Importantly, work carried out in the last two years show that acetylation of proteins is a wide spread phenomenon occurring in a variety of proteins encompassing proteins involved in metabolic pathways, RNA splicing, cell cycle, DNA repair, nuclear proteins, cytoskeleton and nuclear transport (19). One recent study demonstrated in human liver cells (21) that every enzyme involved in glycolysis, fatty acid and glycogen metabolism, tricarboxylic and urea cycles are acetylated. Changes in acetylation status affect the enzymatic activity to allow the cells to respond changes in the metabolic demands (20,21). Thus, modification of proteins through acetylation has been recognized to be as important as modification through phosphorylation (50,51). The finding that aspirin can acetylate multiple proteins suggests that it may exert its chemopreventive effects through alteration of the functional activity of few selective proteins. This represents an important area of future studies.

Acknowledgements

This project was supported by a grant from National Institute of Health (5R03CA133061-02) to G.J.B. We thank Dr Kalkunte Srivenugopal and Dr Vardan Karamyan for advice and useful discussions. We also thank Kevin Welle and the University of Rochester Proteomics Center for mass spectrometry analysis.

References

1. Flossmann E and Rothwell PM: Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet 369: 1603-1613, 2007.
2. Chan AT and Giovannucci EL: Primary prevention of colorectal cancer. Gastroenterology 138: 2029-2043, 2010.
3. Harris RE, Beebe-Donk J, Doss H and Burr Doss D: Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade (review). Oncol Rep 13: 559-583, 2005.
4. Luciani MG, Campregher C and Gasche C: Aspirin blocks proliferation in colon cells by inducing a G1 arrest and apoptosis through activation of the checkpoint kinase ATM. Carcinogenesis 28: 2207-2217, 2007.
5. Hanif R, Pittas A, Feng Y, *et al*: Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem Pharmacol 52: 237-245, 1996.
6. Chan AT, Ogino S and Fuchs CS: Aspirin use and survival after diagnosis of colorectal cancer. JAMA 302: 649-658, 2009.
7. Kopp E and Ghosh S: Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 265: 956-959, 1994.
8. Roth GJ, Machuga ET and Ozols J: Isolation and covalent structure of the aspirin-modified, active-site region of prostaglandin synthetase. Biochemistry 22: 4672-4675, 1983.
9. Vane JR and Botting RM: The mechanism of action of aspirin. Thromb Res 110: 255-258, 2003.
10. Oshima M, Dinchuk JE, Kargman SL, *et al*: Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 87: 803-809, 1996.
11. Soh JW and Weinstein IB: Role of COX-independent targets of NSAIDs and related compounds in cancer prevention and treatment. Prog Exp Tumor Res 37: 261-285, 2003.
12. Yin MJ, Yamamoto Y and Gaynor RB: The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 396: 77-80, 1998.
13. Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY and Vilcek J: Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. Proc Natl Acad Sci USA 94: 2869-2873, 1997.
14. Martinez ME, O'Brien TG, Fultz KE, *et al*: Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. Proc Natl Acad Sci USA 100: 7859-7864, 2003.
15. Caterson RJ, Duggin GG, Horvath J, Mohandas J and Tiller D: Aspirin, protein transacetylation and inhibition of prostaglandin synthetase in the kidney. Br J Pharmacol 64: 353-358, 1978.
16. Green FA and Jung CY: Acetylation of erythrocytic membrane peptides by aspirin. Transfusion 21: 55-58, 1981.
17. Alfonso LF, Srivenugopal KS, Arumugam TV, Abbruscato TJ, Weidanz JA and Bhat GJ: Aspirin inhibits camptothecin-induced $p21^{CIP1}$ levels and potentiates apoptosis in human breast cancer cells. Int J Oncol 34: 597-608, 2009.
18. Alfonso LF, Srivenugopal KS and Bhat GJ: Does aspirin acetylate multiple cellular proteins? (Review). Mol Med Rep 2: 533-537, 2009.
19. Choudhary C, Kumar C, Gnad F, *et al*: Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325: 834-840, 2009.
20. Wang Q, Zhang Y, Yang C, *et al*: Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science 327: 1004-1007, 2010.
21. Zhao S, Xu W, Jiang W, *et al*: Regulation of cellular metabolism by protein lysine acetylation. Science 327: 1000-1004, 2010.
22. Pinckard RN, Hawkins D and Farr RS: *In vitro* acetylation of plasma proteins, enzymes and DNA by aspirin. Nature 219: 68-69, 1968.
23. Warburg O: On the origin of cancer cells. Science 123: 309-314, 1956.
24. Mazurek S: Pyruvate kinase type M2: A key regulator of the metabolic budget system in tumor cells. Int J Biochem Cell Biol (In press).
25. Cairns RA, Harris IS and Mak TW: Regulation of cancer cell metabolism. Nat Rev Cancer 11: 85-95, 2011.
26. Roberts MS, Rumble RH, Wanwimolruk S, Thomas D and Brooks PM: Pharmacokinetics of aspirin and salicylate in elderly subjects and in patients with alcoholic liver disease. Eur J Clin Pharmacol 25: 253-261, 1983.
27. Hitosugi T, Kang S, Vander Heiden MG, *et al*: Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. Sci Signal 2: ra73, 2009.
28. Christofk HR, Vander Heiden MG, Harris MH, *et al*: The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452: 230-233, 2008.
29. Le A, Cooper CR, Gouw AM, *et al*: Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc Natl Acad Sci USA 107: 2037-2042, 2010.

30. Basu A, Rose KL, Zhang J, *et al*: Proteome-wide prediction of acetylation substrates. Proc Natl Acad Sci USA 106: 13785-13790, 2009.
31. Ramos-Montoya A, Lee WN, Bassilian S, *et al*: Pentose phosphate cycle oxidative and nonoxidative balance: A new vulnerable target for overcoming drug resistance in cancer. Int J Cancer 119: 2733-2741, 2006.
32. Boros LG, Lee PW, Brandes JL, *et al*: Nonoxidative pentose phosphate pathways and their direct role in ribose synthesis in tumors: is cancer a disease of cellular glucose metabolism? Med Hypotheses 50: 55-59, 1998.
33. Rowland M, Riegelman S, Harris PA and Sholkoff SD: Absorption kinetics of aspirin in man following oral administration of an aqueous solution. J Pharm Sci 61: 379-385, 1972.
34. Liyasova MS, Schopfer LM and Lockridge O: Reaction of human albumin with aspirin in vitro: mass spectrometric identification of acetylated lysines 199, 402, 519, and 545. Biochem Pharmacol 79: 784-791, 2010.
35. Needs CJ and Brooks PM: Clinical pharmacokinetics of the salicylates. Clin Pharmacokinet 10: 164-177, 1985.
36. Cherian M and Abraham EC: Glycation of human lens crystallins: effect of age and aspirin treatment. Ophthalmic Res 25: 349-354, 1993.
37. Mathupala SP, Ko YH and Pedersen PL: Hexokinase-2 bound to mitochondria: cancer's stygian link to the 'Warburg effect' and a pivotal target for effective therapy. Semin Cancer Biol 19: 17-24, 2009.
38. Zhou W, Capello M, Fredolini C, *et al*: Mass spectrometry analysis of the post-translational modifications of alpha-enolase from pancreatic ductal adenocarcinoma cells. J Proteome Res 9: 2929-2936, 2010.
39. Vizan P, Alcarraz-Vizan G, Diaz-Moralli S, Solovjeva ON, Frederiks WM and Cascante M: Modulation of pentose phosphate pathway during cell cycle progression in human colon adenocarcinoma cell line HT29. Int J Cancer 124: 2789-2796, 2009.
40. Tian WN, Braunstein LD, Pang J, *et al*: Importance of glucose-6-phosphate dehydrogenase activity for cell growth. J Biol Chem 273: 10609-10617, 1998.
41. Board M, Humm S and Newsholme EA: Maximum activities of key enzymes of glycolysis, glutaminolysis, pentose phosphate pathway and tricarboxylic acid cycle in normal, neoplastic and suppressed cells. Biochem J 265: 503-509, 1990.
42. Jiang P, Du W, Wang X, *et al*: p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. Nat Cell Biol 13: 310-316, 2011.
43. Tennant DA, Duran RV and Gottlieb E: Targeting metabolic transformation for cancer therapy. Nat Rev Cancer 10: 267-277, 2010.
44. Boros LG, Puigjaner J, Cascante M, *et al*: Oxythiamine and dehydroepiandrosterone inhibit the nonoxidative synthesis of ribose and tumor cell proliferation. Cancer Res 57: 4242-4248, 1997.
45. Rais B, Comin B, Puigjaner J, *et al*: Oxythiamine and dehydroepiandrosterone induce a G1 phase cycle arrest in Ehrlich's tumor cells through inhibition of the pentose cycle. FEBS Lett 456: 113-118, 1999.
46. Yang Y, Rao R, Shen J, *et al*: Role of acetylation and extracellular location of heat shock protein 90alpha in tumor cell invasion. Cancer Res 68: 4833-4842, 2008.
47. Guzman-Aranguez A, Olmo N, Turnay J, *et al*: Differentiation of human colon adenocarcinoma cells alters the expression and intracellular localization of annexins A1, A2, and A5. J Cell Biochem 94: 178-193, 2005.
48. Lane AA and Chabner BA: Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 27: 5459-5468, 2009.
49. Spange S, Wagner T, Heinzel T and Kramer OH: Acetylation of non-histone proteins modulates cellular signalling at multiple levels. Int J Biochem Cell Biol 41: 185-198, 2009.
50. Kouzarides T: Acetylation: a regulatory modification to rival phosphorylation? EMBO J 19: 1176-1179, 2000.
51. Norvell A and McMahon SB: Cell biology. Rise of the rival. Science 327: 964-965, 2010.