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Within the DCBMO, two partially autonomous research groups were involved in specifically oriented research. The group **"Analysis of proteins important in biomedicine"** leaded by Prof. Emil Paleček dealed mainly with peptides and proteins and particularly with their properties at electrically charged surfaces (mostly concentrated in field II, as specified below). The research was oriented toward a new method of electrochemical analysis based on the ability of proteins to catalyze hydrogen evolution at mercury electrodes. Such electrocatalysis is manifested by the so-called peak H, yielded by constant current chronopotentiometric stripping method. Peak H differs from the previously studied electrochemical signals of proteins particularly (i) by its ability to detect proteins down to nanomolar and subnanomolar concentrations and (ii) by its high sensitivity (a) to local and global changes in protein structures and (b) to protein redox states. In 2009 a considerable progress in electrochemical analysis of proteins, and particularly in the studies of changes in the protein structure at electrically charged surfaces was achieved. In addition, the group focused on the electrochemical analysis and chemical modification of polysaccharides.

The group **"Physics and Physical Chemistry of Biopolymers"** leaded by Dr. František Jelen was oriented towards (a) interactions of nucleic acids components with metal ions, such as copper; (b) development of electrochemical methods for microanalysis of nucleic acids components, their metabolites and analogues; (c) application of elimination voltammetry (EVLS) in analysis of nucleic acids. EVLS in connection with the stripping procedure proved useful for both qualitative and quantitative microanalysis of purine derivatives, and can reveal details of studied electrode processes. Activities of the group came mainly under the field I.

Summary of the results:

In 2009 the Department of Biophysical Chemistry and Molecular Oncology pursued research concentrated to three main fields (see below). Despite existence of the above mentioned groups, many results arose from collaboration of scientists through the whole Department.

Field I: Electrochemistry of natural, synthetic and chemically modified nucleic acids and their components, development of electrochemical DNA sensors and their applications in detection of DNA damage, DNA hybridization and in molecular diagnostics

Field II: Properties of peptides, proteins and polysaccharides at electrically charged surfaces, application of electrochemistry in development of novel micromethods for protein and polysaccharide analysis

Field III: Structure and interaction of DNA and proteins in oncological research, especially with respect to the p53-family proteins

Research in the Field I included systematic studies of the behavior of nucleic acids components, their metabolites, metal complexes, synthetic oligo-nucleotides (ODNs), chemically modified or damaged DNAs and their complexes with biologically active compounds at electrodes. The studies were oriented towards novel techniques of electrochemical DNA labeling and development of new bioanalytical and diagnostic approaches applicable in practical biosensing.

Adsorption and two-dimensional condensation of 5-methylcytosine

Purine and pyrimidine derivatives occurring in nucleic acids posses an extraordinary high ability of self-association at the electrode surface and can form there by a two-dimensional (2D) condensation a monomolecular compact film (self-assembled monolayer - SAM). The effects of methyl substituent on the 2D condensation were studied using the 5-methylcytosine molecule which is involved in gene silencing and has a great biological impact. At acid pHs, 5-methylcytosine forms at the mercury electrode a physisorbed self-assembled 2D layer at potentials close to the potential of electrocapillary maximum. From the temperature dependence of the electrode double layer capacitance, the standard Gibbs energy of adsorption ($\Delta G_m \tilde{=} .7$ kJmol^{-1}), lateral interaction coefficient of the Frumkin adsorption isotherm (ac=2.05) and area occupied by one molecule (A=1.31 nm^2) in the 2D layer were determined. Measurements performed on a single-crystal Au(111) surface show that the 2D condensation can take place on other substrates as well.

Improved electrochemical detection of purine nucleobases at mechanically roughened edge-plane pyrolytic graphite electrode

Mechanically grinded edge-plane pyrolytic graphite electrode (g-PGEe) was applied in voltammetric analysis of purine nucleobases, acid-hydrolyzed synthetic oligodeoxynucleotides and a nonhydrolyzed plasmid DNA. Properties of the mechanically grinded electrodes in these analytical applications were compared with some other carbon electrode types. We show that the electrode surface grinding with 15-μm SiC particles resulted in a remarkable improvement of oxidation signals of purine bases with no addition of copper ions. Addition of the copper ions, causing a strong enhancement of the purine oxidation responses at fine-polished carbon electrodes, had only small effect on the purine signals at the g-PGEe. On the other hand, the g-PGEe appeared less suitable for the ex situ AdTS voltammetric measurements of nonhydrolyzed plasmid DNA, compared to freshly peeled basal plane pyrolytic graphite electrode.

Improved sensitivity and selectivity of uric acid voltammetric sensing with mechanically grinded carbon/graphite electrodes

Determination of uric acid (UA) levels in body fluids is important for diagnostics and prevention of severe metabolic disorders. Electrochemical determination of the UA relies on an oxidation signal measurable at different carbon-based electrodes. Improvement of the UA electrochemical sensing has usually been attained via various modifications of the electrode surfaces. We showed that a strong enhancement of the UA oxidation

signal can be reached by a simple mechanical grinding of the surfaces of glassy carbon or edge plane-oriented pyrolytic graphite electrodes with SiC particles of an optimum size 15µm. In contrast to fine polished electrodes (finally with 1-µm particles), the grinded ones exhibited an excellent separation of oxidation signals of ascorbic acid, dopamine (representing most important natural interferents in UA determination), xanthine and hypoxanthine (precursors of UA in purine catabolism), making it possible to detect these substances in a mixture. Enhancement of UA and dopamine (DA) oxidation signals at the grinded electrodes allowed their easy detection at nanomolar levels in up to 104-fold excesses of ascorbic acid. Due to a strong adsorption at the electrode surface, nanomolar concentrations of UA and DA can be determined by ex situ voltammetry. Similarly, strong enhancement of oxidation signals was observed for purine nucleobases, guanine and adenine. The grinded electrodes have been tested in analysis of real clinical samples of human serum or urine. An excellent agreement between electrochemical and routine biochemical determination of UA in the biological samples is demonstrated.

Simultaneous electrochemical monitoring of metabolites related to the xanthine oxidase pathway using a grinded carbon electrode

Using a mechanically grinded pyrolytic graphite electrode in edge orientation, a sensitive electrochemical method was developed for simultaneous determination of uric acid (UA), xanthine (XAN), hypoxanthine (HYP) (products of purine catabolism in human), allopurinol (ALO), and oxypurinol (OXY) (a drug used in treatment of purine catabolism disorders and its metabolite, respectively). It is demonstrated that differential pulse voltammetry in connection with this electrode can serve as a simple and efficient tool for monitoring transformation of purine catabolites (HYP → XAN →UA) catalyzed by xanthine oxidase (XO) as well as inhibition of this pathway by ALO being enzymatically converted to OXY. Our protocol is based on direct electrochemical measurement of oxidation peaks for each of the substances during in vitro reactions in a single detection step by the same electrode system. In addition, we show that the proposed electrochemical technique can be applied to parallel detection of metabolites involved in the XO pathway excreted in urine without any pretreatment of the clinical samples.

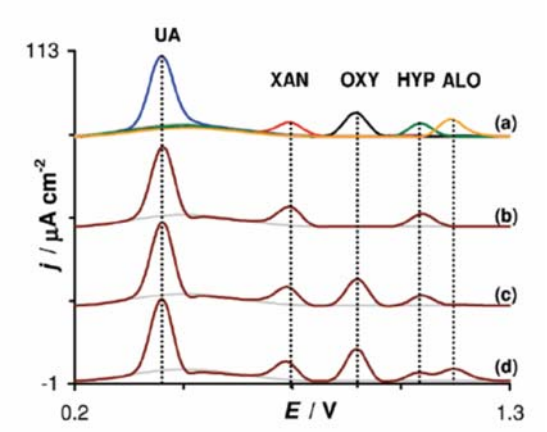

Figure 1: Electrochemical responses of uric acid (UA), xanthine (XAN), hypoxanthine (HYP), allopurinol (ALO) and oxypurinol (OXY) at a grinded graphite electrode (from top to bottom: individual substances and mixtures UA+XAN+HYP, UA+XAN+HYP+OXY, UA+XAN+HYP,+OXY+ALLO).

Voltammetric study of adenine complex with copper on mercury electrode

The determination of adenine (Ade), adenosine (Ado) and hydrolyzed adenosine (h-Ado) in the presence of copper(II) ions is described by cyclic (CV) and elimination voltammetry with linear scan (EVLS) in connection with adsorptive stripping technique. Signals of adenine and copper-adenine complex were measured on a hanging mercury drop electrode (HMDE) in buffered solutions with different pH. The differences in electrochemical behavior of Ade and Ado were found not only in dependence on the presence of copper ions, scan rate, Ade concentration and pH, but also on the accumulation time and potential where a copper-Ade complex is formed. A deeper evaluation of voltammetric responses was carried out by EVLS. The EVLS function E4 eliminating charging and kinetic current components and conserving the diffusion current component was capable of enhancing the current sensitivity of CV peaks and of detecting electron transfer in adsorbed state. The irreversible electrode process of a totally adsorbed electroactive species is indicated by

means of a peak-counterpeak signal. Our results show that EVLS in connection with the adsorptive stripping procedure is not only a useful tool for both qualitative and quantitative microanalysis of Ade but also for revealing certain details in electrode processes.

Detection of abasic sites in DNA by electrochemical, immuno-electrochemical and acoustic methods using OsO_4, 2,2 '-bipyridine as a probe for unpaired thymine residues

We report on comparative analysis of the detection of DNA damage modeled by presence of abasic sites (AP) at defined positions using the chemical modification of the DNA by the complex of osmium tetroxide-2,2-bipyridine (Os,bipy) that selectively binds to unpaired thymine residues in the damaged DNA. AP were detected by electrochemical detection (EC) of the Os,bipy-thymine adducts, by immunoelectrochemical (IE) and by thickness shear mode acoustic methods (TSM). EC method of detection can perfectly distinguish between the number of AP. IE and TSM methods were of comparable sensitivity.

End-labeling of peptide nucleic acid with osmium complex. Voltammetry at carbon and mercury electrodes

Peptide nucleic acid (PNA), the DNA mimic with electrically neutral pseudopeptide backbone, is intensively used in biotechnologies and particularly in single-base mismatch detection in DNA hybridization sensors. We propose a simple method of covalent end-labeling of PNA with Os,bipy. Os,bipy-modified PNA (PNA-Os,bipy) produces voltammetric stripping peaks at carbon and mercury electrodes. Peak potential (Ep) of one of the anodic peaks of PNA-Os,bipy at the pyrolytic graphite electrode (PGE) differs from Ep of the reagent, allowing PNA-Os,bipy analysis directly in the reaction mixture. At the hanging mercury electrode (HMDE) the PNA-Os,bipy yields a catalytic peak Catp, in addition to the redox couples. Using Catp it is possible to detect purified PNA–Os,bipy down to 1 pM concentration at accumulation time 60 s. To our knowledge this is the highest sensitivity of the electrochemical detection of PNA.

Electrochemical DNA detection based on the polyhedral boron cluster label

Polyhedral boron clusters are proposed as new, chemically and biologically stable, versatile redox labels for electrochemical DNA hybridization sensors. Selective and sensitive detection of the redox labeled DNA-probe was achieved by means of covalently attached electroactive marker 7,8-dicarba-nido-undekaborate group. A nanomolar concentration of boron cluster-labeled DNA was recognized. High specificity of the analysis with the boron cluster-labeled DNA probe, including detection of single base mismatch, was demonstrated. The above findings, together with proposed earlier use of metallacarboranes as an electrochemical label for biomolecules opens the door for a “multicolor” electrochemical coding of DNA with boron clusters and simultaneous detection of several DNA targets.

Detection of single nucleotide polymorphisms in p53 mutation hotspots and expression of mutant p53 in human cell lines using an enzyme-linked electrochemical assay

An enzyme-linked electrochemical technique for single nucleotide polymorphism (SNP) typing in the p53 tumor suppressor gene is presented. The technique is based on a DNA polymerase-catalyzed extension of a primer hybridized to a target DNA strand upstream (5’→3’) to the SNP site by one nucleotide bearing a biotin tag. Under optimized conditions, efficient incorporation of the biotinylated nucleotide occurs only in the case of complementarity between the first nucleotide in single-stranded 5’-overhang of the target strand. The introduced biotin tag is detected after capture of the primer extension products at magnetic beads bearing oligoT strands via oligoA adaptors at 5’-ends of the primer, binding of streptavidin-alkaline phosphatase conjugate and enzymatic conversion of 1-naphthyl phosphate into 1-naphthol which is determined electrochemically at carbon electrodes. In addition to model studies with synthetic oligonucleotides, we report on detection of mutant p53 expression in human cell lines using reverse transcription-PCR technique combined with amplified primer extension and the magnetic beads-based electrochemical assay.

Tetrathiafulvalene-labelled nucleosides and nucleoside triphosphates: synthesis, electrochemistry and the scope of their polymerase incorporation into DNA

The title 5-substituted pyrimidines (U and C) and 7-substituted 7-deazapurines (7-deazaA and 7-deazaG) bearing tetrathiafulvelene (TTF) attached through an acetylene linker have been prepared by Sonogashira cross-coupling of the corresponding 5- or 7-iodo derivatives of nucleosides with 2-ethynyltetrathiafulvalene. Their subsequent triphosphorylation gave the corresponding nucleoside triphosphates (dNTPs). Square-wave voltammetry of the TTF-labeled nucleosides and nucleotides showed two peaks, one at 0.2–0.3 V and the other at around 0.65 V (vs. Ag|AgCl|3 M KCl), which correspond to two reversible one-electron redox.

Base-modified DNA labeled by [Ru(bpy)(3)](2+) and [Os(bpy)(3)](2+) complexes: construction by polymerase incorporation of modified nucleoside triphosphates, electrochemical and luminescent properties, and applications

Modified 2'-deoxynucleoside triphosphates (dNTPs) bearing $[Ru(bpy)_3]^{2+}$ and $[Os(bpy_3)]^{2+}$ complexes attached via an acetylene linker to the 5-position of pyrimidines (C and U) or to the 7-position of 7-deazapurines (7-deaza-A and 7-deaza-G) have been prepared in one step by aqueous crosscouplings of halogenated dNTPs with the corresponding terminal acetylenes. Polymerase incorporation by primer extension using Vent (exo-) or Pwo polymerases gave DNA labeled in specific positions with Ru^{2+} or Os^{2+} complexes. Square-wave voltammetry could be efficiently used to detect these labeled nucleic acids by reversible oxidations of $Ru^{2+/3+}$ or $Os^{2+/3+}$. The redox potentials of the Ru^{2+} complexes (1.1–1.25 V) are very close to that of G oxidation (1.1 V), while the potentials of Os^{2+} complexes (0.75 V) are sufficiently different to enable their independent detection. On the other hand, Ru^{2+}-labeled DNA can be independently analyzed by luminescence. In combination with previously reported dNTPs bearing ferrocene, aminophenyl, and nitrophenyl tags, the Os-labeled dATP has been successfully used for "multicolor" redox labeling of DNA and for DNA minisequencing.

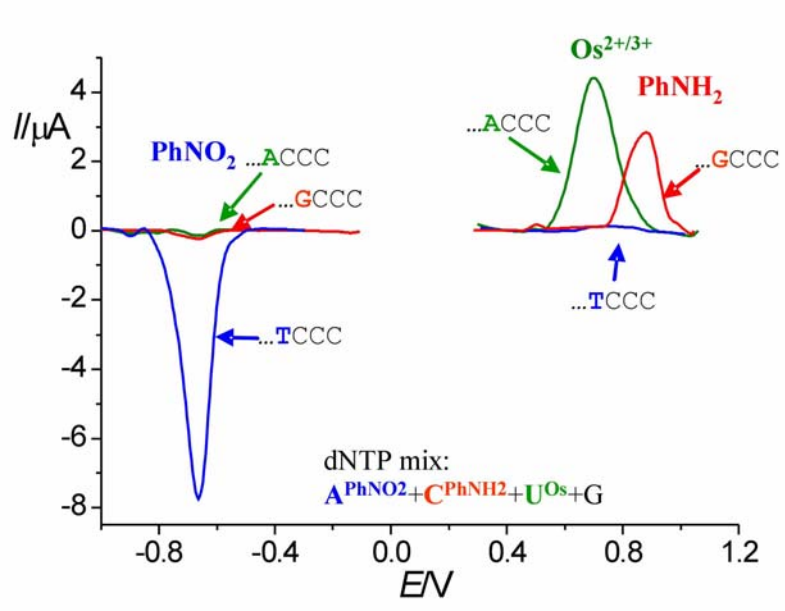

Figure 2: Electrochemical DNA minisequencing using nucleotides labeled with nitrophenyl (PhNO2), aminophenyl (PhNH2) and $[Os(bpy)_3]^{2+}$ (Os).

Ex situ voltammetry and chronopotentiometry of doxorubicin at a pyrolytic graphite electrode: redox and catalytic properties and analytical applications

Ex situ (adsorptive transfer stripping) electrochemical techniques in connection with basal-plane PGE have been applied to the study of redox and catalytic properties of doxorubicin (DOX). Cyclic and square-wave voltammetry and constant current chronopotentiometric stripping (CPS) analysis were used to follow reversible reduction of DOX quinone moiety around -0.5 V and its coupling to catalytic oxygen reduction. CPS was for the first time used for sensitive ex situ determination of the DOX using the catalytic signal around -0.5 V in the presence of oxygen, allowing detection of femtomole amounts of DOX. We show that specific interaction of DOX with double-stranded DNA can easily be monitored using the catalytic CPS signal.

Indicator-based and indicator-free magnetic assays connected with disposable electrochemical nucleic acid sensor system

An indicator-based and indicator-free magnetic assays connected with a disposable pencil graphite electrode (PGE) were successfully developed, and also compared for the electrochemical detection of DNA hybridization. The oxidation signals of echinomycin (ECHI) and electroactive DNA bases, guanine and adenine, respectively were monitored in the presence of DNA hybridization by using differential pulse voltammetry (DPV) technique. The biotinylated probe was immobilized onto the magnetic beads (magnetic particles, microspheres) and hybridization with its complementary target at the surface of particles within the medium was exhibited successfully using electrochemical sensor system. For the selectivity studies, the results represent that both indicator-based and indicator-free magnetic assays provide a better discrimination for DNA hybridization compared to duplex with one-base or more mismatches. The detection limits (S/N = 3) of the magnetic assays based on indicator or indicator-free systems were found in nM concentration level of target using disposable sensor technology with good reproducibility. The characterization and advantages of both proposed magnetic assays connected with a disposable electrochemical sensor are also discussed and compared with those methods previously reported in the literature.

In the Field II the work included basic studies of electrochemical behavior of peptides and proteins. Efficient, highly sensitive electrochemical techniques suitable for monitoring protein denaturation and determination of redox state, interactions of apoproteins with their cofactors. Potential-dependent changes in the structure of a protein adsorbed at mercury surface were observed for the first time. Osmium tetroxide complexes were introduced as new tools for electrochemical analysis of polysaccharides and new procedures for electrode pretreatment were applied.

Electrochemical determination of thioredoxin redox states

Thioredoxin (TRX) is a general protein disulfide reductase with a large number of biological functions, including its roles in human diseases. The TRX redox mechanism is based on reversible oxidation of two cysteine thiol groups to a disulfide, accompanied by the transfer of two protons. Using constant-current chronopotentiometric stripping analysis (CPSA) and the electrocatalytic TRX peak H, we have determined redox states of TRX at submicromolar TRX concentrations. A concentration of 1 nM TRX produces a well-developed peak H at moderate accumulation time without stirring. On the basis of this peak, interactions of 4-hydroxy-2-nonenal (HNE, product of lipid peroxidation) with TRX and the formation of TRX-HNE adducts were studied. CPSA of TRX at a carbon electrode is less sensitive and does not discriminate between reduced and oxidized forms of TRX.

Ionic strength-dependent structural transition of proteins at electrode surfaces

Using constant current chronopotentiometry we showed that in 50 mM sodium phosphate (pH 7) bovine serum albumin and some other proteins were not significantly denatured at a bare mercury electrode while at higher phosphate concentrations they underwent electric field-driven denaturation on the electrode surface.

Voltammetry of Os(VI)-modified polysaccharides at carbon electrodes

We show that polysaccharides (PSs, such as dextran and mannan) can be chemically modified by Os(VI) complexes, yielding electroactive adducts. Os(VI) complexes with different ligands (e.g., temed and 2,2'-bipyridine) produced at pyrolytic graphite electrodes redox couples at different potentials suitable for "multicolor" labeling of PSs and for studies of ligand exchange kinetics. PS-Os(VI)L adducts can be determined not only in their purified forms but also in the reaction mixtures.

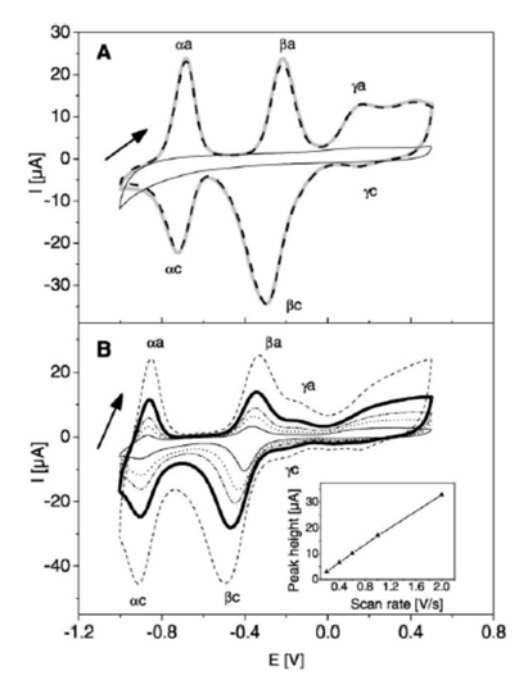

Figure 3: Electrochemical responses of Os(VI),L-modified dextran at a carbon electrode.

Electrochemical renewal of stationary mercury drop or meniscus electrodes

We show that a liquid mercury electrode surface can be electrochemically renewed without mechanical detachment of the drop. Voltammetric experiments with a mechanically renewed stationary (hanging) mercury drop or meniscus electrode (SME) and an electrochemically renewed SME are compared. The measurements were performed with two surface active organic depolarizers, i.e., 2-aminoanthraquinone and dithiothreitol and surface inactive $Cd(NO_3)_2$. The results show that efficient purely electrochemical renewal of the electrode surface of SME for voltammetric purposes is possible.

Interaction of biomacromolecules with surfaces viewed by electro-chemical methods

The electrocatalytic evolution of hydrogen on mercury electrodes by organic molecules indicates, when followed with chronopotentiometric stripping method by the „peak H", that when the stripping current is changed, the mechanism of the electrode process changes. The changes become the more prominent the larger is the catalyzing organic molecule. We explain this phenomenon by dynamic interaction of the catalyzing molecule with the electrode surface.

In the Field III, the studies on structure and interactions of the proteins involved in important signaling pathways were continued. Modulation of gene expression in U251 glioblastoma cells by binding of mutant p53 R273H to intronic and intergenic sequences was described. Effects of DNA supercoiling on DNA binding by BRCA1 protein was reported for the first time. Bilateral changes in IL-6 protein, but not in its receptor gp130, in rat dorsal root ganglia following sciatic nerve ligature, were observed.

Modulation of gene expression in U251 glioblastoma cells by binding of mutant p53 R273H to intronic and intergenic sequences

Missense point mutations in the TP53 gene are frequent genetic alterations in human tumor tissue and cell lines derived thereof. Mutant p53 (mutp53) proteins have lost sequence-specific DNA binding, but have retained the ability to interact in a structure selective manner with non-B DNA and to act as regulators of transcription. To identify functional binding sites of mutp53, we established a small library of genomic sequences bound by p53 R273H in U251 human glioblastoma cells using chromatin immunoprecipitation (ChIP). Mutp53 binding to isolated DNA fragments confirmed the specificity of the ChIP. The mutp53-bound DNA sequences are rich in repetitive DNA elements, which are dispersed over non-coding DNA regions. Stable down-regulation of mutp53 expression strongly suggested that mutp53 binding to genomic DNA is functional. We identified the PPARGC1A and FRMD5 genes as p53 R273H targets regulated by binding to intronic and intra-genic sequences. We propose a model that attributes the oncogenic functions of mutp53 to its ability to interact with intronic and intergenic non-B DNA sequences and modulate gene transcription via re-organization of chromatin.

The central region of BRCA1 binds preferentially to supercoiled DNA

BRCA1 is a multifunctional tumor suppressor protein with implications in regulating processes, such as cell cycle, transcription, DNA repair, and chromatin remodeling. The function of BRCA1 likely involves interactions with a vast number of proteins and likewise DNA. To this date there is only fragmentary evidence about BRCA1 binding to DNA. In this study, we provide detailed analyses of various BRCA1 protein constructs binding to linear and supercoiled (sc) DNAs. We demonstrate that the central region of human BRCA1 binds strongly to negatively sc plasmid DNA at a native superhelix density, as evidenced by electrophoretic retardation of sc DNA in agarose gels. At relatively low BRCA1:DNA ratios, binding of BRCA1 to sc DNA results in the appearance of one or more retarded DNA bands on the gels. After removal of BRCA1, the original mobility of the sc DNA is recovered. BRCA1 proteins at higher concentrations also bind to the same DNA but in linear state, leading to formation of a smeared retarded band. Our experiments not only demonstrate a preference for BRCA1 binding to sc DNA, but also show that the central region may contain at least two efficient DNA binding domains with strong affinity for sc DNA. The biological implications of the novel DNA binding activities of BRCA1 are discussed.

Bilateral changes in IL-6 protein, but not in its receptor gp130, in rat dorsal root ganglia following sciatic nerve ligature

Local intracellular signaling cascades following peripheral nerve injury lead to robust axon regeneration and neuropathic pain induction. Cytokines are classic injury-induced mediators. We used sciatic nerve ligature (ScNL) to investigate temporal changes in IL-6 and its receptor gp130 in both ipsilateral and contralateral lumbal (L4-L5) dorsal root ganglia (DRG). Rats were operated aseptically on unilateral ScNL and allowed to survive for 1, 3, 7, and 14 days. Immunohistochemistry and Western blot analysis were used to determine levels of IL-6 and gp130 in DRG. A distinct increase in immunostaining for IL-6 was found in the neuronal cell bodies of sections through both ipsilateral and contralateral DRG at 1 and 3 days after operation. After 7 and 14 days, the DRG sections displayed only a moderate elevation in immunostaining when compared with sections of naïve DRG. The levels of IL-6 protein increased in both ipsilateral and contralateral lumbal DRG following peripheral nerve injury. The elevation of IL-6 protein was significant in both ipsilateral and contralateral DRG 1, 3, 7, and 14 days after operation. On the other hand, the levels of gp130 receptor did not change significantly. The data provide evidence for changes in IL-6 levels not only in the DRG associated with the damaged nerve but also in those unassociated with nerve injury during the experimental neuropathic pain model.