REVERSIBLE MICROWAVE EFFECTS ON THE BLOOD–BRAIN BARRIER

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SUMMARY

Low level microwave exposure of Chinese hamsters resulted in reversible permeability of the blood–brain barrier (BBB) to horseradish peroxidase (HRP). Lesions were grossly visible in random areas of the brain immediately following exposure, but were not as common following a 1 h recovery period and were absent after a 2 h recovery period. The apparent route of increased permeability was via endothelial vesicular transport, since reaction product was not seen passing through the endothelial tight junctions. In addition, endothelial flooding of HRP, platelet aggregation and perivascular edema were observed only in experimental animals. Possible mechanisms for the enhanced vesicular transport are discussed.

INTRODUCTION

The existence of a blood–brain barrier was first recognized by Erlich in 1887 and later by Lewandowsky and Goldmann (see review by Davson11). They found that vital dyes injected into the bloodstream stained all the tissues of the animal except the central nervous system. This concept has since been studied extensively utilizing physiological and morphological techniques. According to current concepts, the blood–brain barrier exists throughout the central nervous system except in such selective regions as the median eminence, area postrema, neurohypophysis, subfornical organ and pineal gland10. Some arterioles occasionally have short segments which are normally leaky38. This barrier is not absolute, but consists of regulatory

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mechanisms between the blood and adjacent parenchyma. Undoubtedly, this barrier protects the brain from various neurotoxic substances. In addition, it may be considered as an additional regulator of homeostasis in the brain.

Morphological investigations in search of sites in this barrier have led to the tentative conclusion that at least larger protein tracers are prevented from entering the brain by the capillary endothelium. These investigators have further demonstrated that the site of this barrier probably lies within the endothelial tight junctions. A paucity of pinocytotic vesicles in cerebral vessels is also a contributing factor in this thesis. Similar barrier systems have been demonstrated in the eye, thymus, testes and peripheral nerve.

In several disease and experimental conditions, this barrier becomes more permeable, e.g., anoxia, diabetes, heavy metal poisoning, autoimmune diseases, infusion of hypertonic solution into the brain via the carotid artery and other conditions.

Histopathological changes associated with brain vasculature after non-ionizing microwave irradiation have been observed. These include hemorrhage, congestion and edema, astrocytic and dendritic swelling, vacuolation of neurons, platelet adhesion and endothelial injury associated with the brain vasculature. Frey observed increased fluorescence in microwave irradiated rat brain slices following intravenous injection of fluorescein dye, while Merritt found no change in the amount of fluorescein in rat brains after microwave irradiation. Oscar and Hawkins reported increased amounts of mannitol in rat brains exposed to microwaves compared to controls. Microwave induced hyperthermia at high power densities also increases blood–brain permeability.

These inconsistent results and a general lack of light and/or electron microscopic studies on the effects of microwave exposure on the blood–brain barrier prompted the following investigation concerning the effects of low power density microwaves on the blood–brain barrier of Chinese hamsters. Further, there is a relative absence of data on the mechanism of microwave effects on tissues, but a recognized need for understanding the effects of microwaves on humans and animals due to the rapidly expanding exposure of the American public to industrial and domestic application of microwave technology.

MATERIALS AND METHODS

Thirty-nine Chinese hamsters were irradiated in the far field with microwaves of 2450 MHz (CW) frequency and a power density of 10 mW/cm² for 2 h. The calculated specific absorption rate (SAR) using the radio frequency handbook was 2.5 mW/g. Thirty-nine control animals were sham irradiated (placed in the irradiation chamber but shielded). All exposures were carried out at the Walter Reed Army Institute for Research located in Forest Glenn, Maryland. This power density was chosen because it is considered safe for continuous human exposure in the United States but not in the Soviet Union, China and some European countries. The safe exposure level in these countries is at least an order of magnitude lower than that of the United States.
Immediately following irradiation, the animals were anesthetized with Nembutal and 10 mg of horseradish peroxidase (HRP, Sigma type VI) in 0.2 ml saline was injected into the femoral vein. After 5 min, each animal was perfused transcardially with 1% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 followed by buffered 4% glutaraldehyde for 5 min each. Animals were left overnight at 4 °C prior to dissection of the brain, tissue blocks were washed in 0.1 M cacodylate buffer before and after 100 μm sections were cut on a TC-2 tissue chopper. Sections were incubated for 45 min at room temperature with agitation in a medium consisting of 5 mg of 3,3'-diaminobenzidine (DAB) in 10 ml Tris buffer (pH 7.6) with the addition of 0.1 ml of 1% H₂O₂. After an overnight buffer rinse, sections were evaluated by two individuals independently with a dissection microscope for grossly visible leakage of HRP. The regional frequency of the lesions and the color intensity of the reaction product were subjectively integrated and scored on a scale of 1 to 4. A score of 4 represented multiple, dark reaction sites in a discrete region of the brain and a score of 1 represented at least one pale brown lesion in a specific region. Lesion areas were further trimmed, postfixed in 2% OsO₄ in cacodylate buffer, and stained en bloc in uranyl acetate. Following a graded dehydration in ethanol, sections were embedded in Epon–Araldite for light and electron microscopy. Another series of 12 animals were similarly exposed to microwave irradiation but were allowed to recover for either 1 or 2 h prior to fixation. An equivalent number of control animals were sham irradiated.

RESULTS

Gross observations

A light dark-brown reaction product results when DAB reacts with the HRP in the presence of H₂O₂. Thus, visualization of a brown reaction product in the brain slices was interpreted to be evidence of HRP leakage in a particular area of the brain. This reaction product was observed in the normally leaky areas of the posterior pituitary, median eminence, pineal gland, area postrema, choroid plexus, and the subfornical organ of both control and experimental animals. However, in addition to these leaky areas, similar reaction product was randomly found in several other regions throughout the brains of about one-third of the experimental animals. The lesions seemed to have a random distribution but were most common in the pons, cerebellum, areas around the fourth ventricle and thalamus (Fig. 1). Other areas showing lesions included the hypothalamus, hippocampus and cerebral cortex (Fig. 2). Some control animals also displayed a few random lesion areas with a slight propensity for the thalamus. The reason for the somewhat higher frequency of lesions in the thalamus among control animals is an enigma. However, quantitative scoring on the basis of 1–4 as described under Materials and Methods revealed a considerably higher frequency of lesions in brains of experimental animals.

Light microscopic observations

Survey of unstained thick Epon sections from lesion areas showed that capillaries, venules and some arterioles were often surrounded by distinct brown
Fig. 1. Several areas of HRP leakage are grossly visible in a tissue slice from the thalamus.

Fig. 2. Graph showing the number of animals with grossly visible blood–brain barrier lesions in the experimental and sham irradiated groups. The upper series is from hamsters sacrificed immediately following exposure and the lower series is from hamsters with a 1-h recovery period to show the reversibility effect. Brain regions examined include the cerebral cortex (ctx), hippocampus (hip), thalamus (tha), hypothalamus (hyp), cerebellum (cbl), pons and brain stem areas around the fourth ventricle (a4vr, a4vm, a4cv = rostral, medial, and caudal areas of fourth ventricle).
reaction product, which was absent around the unaffected blood vessels in the adjacent areas of the tissue. Higher magnifications revealed that leaky blood vessels contained HRP in the region of the basal lamina, around pericytes or smooth muscle of the microvasculature and outlining nerve cell bodies (Fig. 3a). The surrounding parenchyma often appeared edematous.

The presence of HRP around leaky vessels and brain parenchyma was confirmed by dark-field microscopy (Fig. 3b), where the HRP reaction product appeared as white granular particles. These particles were concentrated in the vascular walls and the surrounding brain parenchyma of the leaky zones. The concentration of HRP was the greatest near the vessel wall and rapidly decreased peripherally. The area postrema, choroid plexus and neurohypophysis served as controls for dark-field evaluations.

**Electron microscopic observations**

Tissue blocks from the median eminence, neurohypophysis and other selected brain regions which showed leaky vessels upon gross observations were trimmed and thin sectioned. Electron microscopic examination of control and experimental animals revealed that electron dense reaction product formed a thin layer on the luminal
Fig. 4. At the ultrastructural level reaction product is seen along the luminal border of this vessel up to the first tight junction (a), but is absent from the interendothelial space, except by backfilling (b) from the positive basal lamina. HRP is also seen between the perivascular glial endfeet (c) stained section of cerebellum.
surface of the endothelium in many vessels. In addition, the presence of HRP in the basal lamina of normally leaky capillaries and venules of the neurohypophysis as well as in extracellular spaces surrounding these vessels served as a control for the HRP reaction.

Examination of leaky vessels from experimental animals revealed that HRP was present in the vesicular structures of endothelial cells, basal lamina and surrounding pericytes. Tight junctions between endothelial cells appeared intact in all vessels examined and no HRP reaction product could be observed past the first fusion point of the outer leaflets of the endothelial plasma membranes (Figs. 4 and 5). Some reaction product was seen in the interendothelial space on the side of the basal lamina, apparently by backfilling (Figs. 4 and 6). Endothelial cells of irradiated animals appeared to have more pinocytotic vesicles with HRP than in controls (Figs. 6 and 7).

Fig. 8. Reaction product outlines elements of the neuropil, the basal lamina, and the endothelial cytoplasm except for a few clear vacuoles. The capillary lumen appears plugged with the HRP, red blood cells and a platelet (P). Unstained section of the pons.

Fig. 9. The extracellular space (arrowheads) and basal lamina contain HRP reaction product, indicating the leaky nature of this vessel. The lumen contains an aggregation of platelets (P). Stained section of thalamus.

Fig. 5. More reaction product fills the lumen of this vessel but it is absent past the endothelial tight junction. Dense reaction product overlies the basal lamina and extracellular space, as well as the cytoplasmic matrix of the endothelium, except for a few clear vacuoles (arrowheads). Stained section of pons.

Fig. 6. Reaction product is present in the basal lamina, several abluminal vesicles, a coated vesicle (CV), and a short distance back up the interendothelial space (arrowhead). Stained section of hypothalamus.

Fig. 7. Many HRP-filled vesicles are present on the abluminal side of the endothelium and smooth muscle. Two vesicles (arrowhead) appear confluent and the basal lamina is positive. Unstained section of the hypothalamus.
Pinocytotic vesicles containing HRP were often seen opening at the abluminal surface of the endothelial lining and could be traced from the basal lamina to the extracellular spaces of the neuropil (Figs. 5 and 8). When reaction product was seen in the cytoplasm, membrane-bound vacuoles were often clear. Although a few HRP-filled vesicles were confluent, complete transendothelial channels were not seen.

Endothelial cell cytoplasm flooded with HRP was also observed in some leaky vessels (Figs. 5 and 8). This finding was usually accompanied by reaction product in the extracellular space of adjacent neuropil, but sometimes both the vascular lumen and neuropil were completely clear. The endothelium in lesion areas rarely showed advanced stages of necrosis. This phenomenon was never observed in endothelial cells of control animals. In a few lesion areas the involved leaky vessel contained an aggregation of platelets (Figs. 8 and 9). This finding was present despite satisfactory fixation. Occasionally, red blood cells were seen in some incompletely perfused capillaries of both experimental and control animals, but platelet aggregation was never observed in the control animals.

In general, HRP-infiltrated areas of the neuropil exhibited swelling, edema and cytological damage. HRP reaction product was generally not observed in the neuronal and glial cytoplasm, dendrites, axons or their terminals. Perivascular glial endfeet and their associated gap junctions did not appear to retard the passage of HRP into the neuropil. Blood vessels somewhat removed from leaky areas did not display HRP reaction product in their basal laminae or in the surrounding neuropil. Regions lacking reaction product were interpreted to consist of non-leaky, intact blood vessels which were unaffected by microwave irradiation.

**Reversibility experiments**

In the 1 h recovery group only two irradiated animals and one sham-irradiated animal showed evidence of lesions in the brain stem and cerebellum (Fig. 2). None of the animals from the 2 h survival group and their controls had evidence of gross lesions.

**DISCUSSION**

It appears from the above observations that acute irradiation of 2 h duration of Chinese hamsters with 2450 MHz (CW) microwave at a power density of 10 mW/cm² causes reversible alterations in the permeability of the microvasculature in about one-third of the irradiated animals. The lesions were not confined to any particular brain region, although pons, cerebellum, thalamus and areas around the fourth ventricle appeared to be affected most frequently. These results confirm Frey's findings of increased fluorescein extravasation in microwave-irradiated rats, but are contrary to those of Merritt who did not find any increased amounts of fluorescein in brains of animals exposed to microwaves. The discrepancy in these observations could be explained on the basis that Merritt employed 2 mW/cm² power density in contrast to the 10 mW/cm² used in this experiment. However, physiological studies of Oscar and Hawkins showed increased permeability in rat brains after microwave exposure at 2
mW/cm$^2$ power density. Further experiments utilizing multiple isotopes for simultaneous physiological and morphological techniques in the same animals are in progress.

An area of major interest is the route of transport of HRP across the capillary wall to the basal lamina and the extracellular space of the neuropil. It is generally accepted that larger proteins such as HRP are restricted from entering the brain parenchyma due to the presence of tight junctions between endothelial cells and a paucity of pinocytotic vesicles$^{7,8,32}$. Some authors believe that this barrier can be reversibly altered by opening the endothelial tight junctions through cell shrinkage following osmotic manipulation of the blood$^{9,30,31}$ but others suggest alternative routes. Our results indicate little or no transport via interendothelial junctions, since reaction product was not seen past the first luminal tight junction.

Some authors$^{5,15}$ believe that another mechanism of transport through the altered blood–brain barrier is being demonstrated when reaction product floods the endothelial cytoplasm. This finding was occasionally seen following microwave irradiation, but its physiological nature is not clear. The selective permeability of the endothelial cell membrane had undoubtedly broken down, reflecting cell death from either exposure to the microwave irradiation, a toxic effect of the HRP, or ischemia associated with a vascular thrombus. We favor the view$^{5,9,36}$ that this is not a major factor in the breakdown of the blood–brain barrier. The cytoplasmic reaction product seen by Houthoff and Go$^{15}$ was usually associated with a tracer of larger molecular weight and probably lacking in purity. HRP could presumably augment the number of pinocytotic vesicles$^{14}$ but other quantitative evidence denies such an artifact$^{5,21}$. Likewise, we do not believe transendothelial channels are a contributing mechanism, although serial sections were not examined.

The most likely explanation for the increased permeability in the blood–brain barrier following microwave irradiation is suggested by the increase in the number of HRP-filled pinocytotic vesicles in the endothelium of the microvasculature. This has been a common finding in a variety of experimental conditions, including trauma$^{9}$, seizures$^{14,26}$, acute hypertension$^{21}$ and X-irradiation$^{33}$. In all of these cited studies the increase in pinocytotic activity was quantitated at the ultrastructural level. Generally, the increase in the number of vesicles ranged from about 12–16 times the normal value, especially in arterioles. However, direct comparisons are not possible because of differences in the type of lesion, survival time, time of HRP circulation, vessels studied, and manner in which the data is presented. Our preliminary quantitative analysis indicates only a 2–3-fold increase in the number of vesicles in experimental animals.

Mechanisms leading to altered permeability in the blood–brain barrier are not clear. Presman$^{29}$ suggested two possible ways that microwaves may produce biological effects: (1) thermal effects and (2) specific effects not involving heating of tissues. It is generally considered that 10 mW/cm$^2$ does not cause significant heating of tissues. Michaelson$^{20}$ reported less than 1 °C rise in rat colonic temperature after 150 min of whole body exposure to 9 mW/cm$^2$ power density. Our studies on brain temperature measurements after microwave irradiation indicate that at 10 mW/cm$^2$ hyperthermia is not a significant factor. Under the experimental conditions used in this study, we
observed only a 0.4 °C rise in brain temperature. This increase is well within the diurnal cycle of 1–2 °C. Thus, we conclude that the altered blood–brain barrier permeability in these experiments is probably due to some specific, non-thermal effects of microwaves. Although the exact mechanism is still uncertain, several authors have recently implicated the platelet release of serotonin, which triggers cyclic AMP turnover and increases local blood pressure and brain edema through the increase in pinocytotic activity. Other possible chemical factors include norepinephrine, histamine, prostaglandins, glutamate, free radicals and the kallikrein–kininogen–kinin system. Recently, Oscar et al. suggested that the effects of microwave exposure on BBB permeability are related to an increase in the local cerebral blood flow. The mechanical effect of hypertension and vasodilatation may also play a direct or indirect role. That some of these factors might correlate with enhanced vesicular transport following microwave irradiation is suggested by our additional observation of platelet accumulation, parenchymal edema and intact tight junctions, even when the endothelial cytoplasm was flooded with HRP. We also demonstrated that after 2 h of recovery no lesions could be observed. Microwave induced lesions appear to be reversible. A cessation of vesicular activity was also found 2 h following low intensity trauma and the blood–brain barrier was restored even more quickly following electroshock induced seizures. If this reversibility property of altered brain permeability could be controlled and selectively localized, certain applications for cancer therapy may be developed.

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